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IMMUNOCHEMISTRY

BY

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CONTENTS

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INTRODUCTION TO THE CONFERENCE ON IMMUNOCHEMISTRY

By Michael Heidelberger

From the College of Physicians and Surgeons, New York

The Conference on Immunochemistry which I have the privilege and pleasure of now calling to order is the first under the auspices of the Section on Chemistry and Physics of the New York Academy of Sciences to venture so far afield from the sheltered paths of classical physics and chemistry. This excursion is not entirely inappropriate, for if one examines into the matter more closely it becomes evident that Immunochemistry has made its great advances in recent years with the same organic chemical, physical chemical, and analytical chemical methods that have provided the substantial background and material for the other conferences which have been held. While we in Immunochemistry cannot always reason as rigorously as those in the more formal branches of physics and chemistry we are rapidly progressing in this direction and fundamental principles are becoming more clear. The variety and scope of the papers offered guarantee that this forward movement is being continued.

ANTIGENS OF VACCINIA

By Joseph E. Smadel and Theodore Shedlovsky

From the Laboratories and the Hospital of The Rockefeller Institute for Medical Research, New York

The virus of vaccinia is known to have a complex chemical structure. Protein, nucleoprotein, phospholipid, neutral fat, and carbohydrate have been indentified in preparations of purified elementary bodies of vaccinia in amounts comparable, in a general way, to those of bacteria and mammalian cells.[1] The complexity of the virus is also indicated by observations of another sort; for example, a number of different immune bodies are found in the sera of animals following infection with this agent. These antibodies include neutralizing substances,[2] antibodies against a heat-labile (L) and a heat-stable (S) soluble substance,[3] an agglutinin designated "X",[4] and finally, an antibody against a nucleoprotein which is present in elementary bodies.[5]

The nature of the substance or substances in elementary bodies responsible for inducing immunity and the development of neutralizing

antibodies in animals is not known. We shall review briefly the immunological properties of the other antigens of vaccinia and shall discuss at length the relationship that exists between the heat-labile and heat-stable soluble substances. A summary of the work which led to the recognition of the nucleoprotein extracted from elementary bodies as a new antigen of vaccinia[5] will serve as an adequate review of the previously recognized antigens.

About half the material in a dried, purified preparation of elementary bodies of vaccinia is brought into solution by treatment with N/20 NaOH at 56° C. for 15 minutes. The undissolved portion consists of non-infectious ghosts of elementary bodies which have a lower density by ultracentrifugation studies, which stain less deeply by the silver technique, and which appear less brilliant by dark-field illumination than do active virus particles. These particles can be separated from the soluble extracted material by ultracentrifugation at 30,000 r.p.m. A non-infectious solution obtained in this manner by alkaline extraction of virus is rich in a nucleoprotein that contains 14.5 per cent nitrogen and 6 per cent nucleic acid of the thymus type. Electrophoresis studies show that the nucleoprotein constitutes about 90 per cent of the material in the extracts (FIGURE 1) and that this major constituent has a mobility

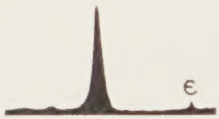

FIGURE 1. Electrophoretic pattern of alkaline extract.

value of 6.4×10^{-5} cm./sec. per volt/cm. in 0.05μ (ionic strength) veronal buffer solution at pH 8.75. The nucleoprotein is soluble at pH values above 8.0 and is partially or completely insoluble in the pH range between 4.5 and 7.5. Serological studies on the nucleoprotein have been carried out in solutions buffered at pH 8.6.

Precipitins which react with this nucleoprotein of vaccinia have been demonstrated in sera of members of several species of animals following hyperimmunization with active virus. Furthermore, rabbits repeatedly injected with inactivated virus particles or with non-infectious alkaline extracts of virus also develop precipitins for the nucleoprotein antigen (NP). Absorption experiments of the type summarized in TABLE 1 clearly indicate that NP-antibodies differ from L-, S-, and X-antibodies of vaccinia. From the results presented in the table it is apparent that hyperimmune rabbit serum precipitated with solutions containing LS-,

TABLE 1

DEMONSTRATION OF MULTIPLE ANTIBODIES IN VACCINAL ANTISERUM

Hyper-immune rabbit serum Absorbed with	Test antigen	Precipitin reactions with solutions of antigen						Agglutinin reactions with suspensions of virus								
		Dilution of test antigen						Dilution of serum								
		1:4	1:8	1:16	1:32	1:64	1:128	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Unabsorbed	LS	++++	++++	++++	+++	+++	++	++++	++++	++++	++++	+++	++	++	+	±
	S	++++	++++	+++	++	+	−									
	NP	++++	++++	++++	+++	++	+									
S-antigen	LS	++++	++++	++++	++++	+++	++									
	S	−	−	−	−	−	−									
	NP	++++	++++	++++	+++	++	±									
LS-antigen	LS	−	−	−	−	−	−	++++	++++	++++	++++	+++	++	+	−	−
	S	−	−	−	−	−	−									
	NP	++++	++++	++++	+++	++	+									
NP-antigen	LS	++++	++++	++++	++++	+++	++	++++	++++	++++	+++	++	+	−	−	−
	S	±	+	+++	++	−	−									
	NP	−	−	−	−	−	−									
LS- and NP-antigens	LS	−	−	−	−	−	−	++++	++++	++++	+++	++	−	−	−	−
	S	−	−	−	−	−	−									
	NP	−	−	−	−	−	−									

A 1:8 dilution of serum no. 1601 used throughout precipitin tests.
E.B. lot no. 1995 diluted 1:6 used throughout agglutination tests.

S-, and NP-antigens and that it agglutinated suspensions of elementary bodies. Absorption with S-antigen removed S-precipitins but left undiminished the L- and NP-antibodies. Similarly, absorption with LS-antigen resulted in a loss of L- and S-antibodies but did not affect the NP-antibodies; the agglutinating titer of this absorbed serum was reduced. In a like manner, the hyperimmune serum after treatment with NP-antigen no longer precipitated with this substance but still contained L- and S-antibodies and still agglutinated the virus. Finally, when removal of all demonstrable precipitins was accomplished by absorption with LS and NP there still remained some agglutinins for the virus particles. Since each of the precipitating antibodies is capable of aggregating elementary bodies the residual agglutinating substance left after removing the precipitins may be designated X-agglutinin. The neutralizing power of the unabsorbed hyperimmune serum, of the portion absorbed free of NP-antibodies, and of the portion absorbed free of all precipitins was essentially the same. Thus it appears that neutralizing antibody is distinct from NP-precipitin; earlier work[6] had indicated that L- and S-antibodies have little or no neutralizing capacity. Further evidence for considering that the nucleoprotein, in its present form at least, is not the substance responsible for the production of immunity, or of neutralizing antibody may be drawn from the experiment in which normal rabbits were injected with solutions of nucleoprotein. All of the animals failed to develop either immunity or appreciable amounts of neutralizing substances, although they did develop precipitins for the antigen and some agglutinins for the virus.

A problem which has intrigued workers in the field of vaccinia is the relationship which exists between the two non-infectious soluble antigens of vaccinia, L and S. That these two antigens are closely associated immunologically was recognized by Craigie and Wishart[3] several years ago. They observed that the absorption of dermal filtrate, which is rich in L- and S-antigens, with either L- or S-antibody removed completely both of the serologically active substances. Because of these findings, Craigie and Wishart[3] stated that "the L and S antigens are different antigenic components of a complex L-S antigen rather than two independent antigens." Under certain conditions, however, results of a different type have been obtained in absorption experiments; for example, removal of all demonstrable S-antigen without complete loss of L-antigen has been effected by treating dermal filtrate with S-antibody (Craigie and Wishart,[4] Parker[7]). It should be emphasized that this latter observation is an exception to the rule, and that such results are not regularly reproducible. To account for this fact, Craigie[4] assumed that under

certain conditions the L-S complex might be dissociated into separate L- and S-fractions.

More recently, Smadel and Rivers[8] observed that heated dermal filtrate was capable of specifically inhibiting L-antibody. (Gentle heating destroys the specific precipitability of L-antigen, but does not affect the serological activity of S-antigen.) Subsequently, it was found that other procedures, which resulted in the inactivation of L-antigen without apparent loss of S-antigen, likewise gave preparations which specifically inhibited the L-antibody. Moreover, the protein-like S-antigen was carefully purified by the technique of Parker and Rivers[9] and even this material was shown to inhibit L-antibody. At this time it was also found that purified S-antigen which had been heated in the presence of dilute alkali lost its ability to precipitate with S-antibody as well as its power to inhibit L-antibody, but that this degraded product was still capable of inhibiting S-antibody. On the basis of such serological evidence these authors[8] suggested that L- and S-antigens probably always occurred together in nature in the form of a single substance with two serologically active parts each of which could be degraded independently of the other. For the experiments of others[4, 7] which had been interpreted as indicating dissociation of the complex into free L and S they offered a different explanation. This was based on the presence in certain dermal filtrates of a hypothetical degraded form of the complex antigen which had a native L-portion and a degraded S-portion[8].

Studies on the relationship of L- and S-antigens were hampered by the fact that neither L- nor LS-complex had been isolated in pure form. Comparatively pure preparations of S-antigen had been obtained but it remained to be proved conclusively that the inhibitory power of this protein for L-antibody was dependent upon the presence of a degraded L-portion of LS-complex, or that free L-antigen, in a partially denatured state, was present as a contaminant[8]. A method for concentrating and partially purifying both L- and S-antigens from dermal filtrate has been reported by Craigie and Wishart[3] who found that these antigens could be precipitated at pH 4.5 and redissolved at pH 6.5. This method, with only minor modifications, has been successfully employed by us. The principal change we have made is to concentrate the filtrate ten to twenty times by evaporation through cellophane sausage casing before precipitating the antigens by dialysis against buffer solution at pH 4.5.

Electrophoretic studies in which the moving boundary apparatus of Tiselius was employed were made on whole concentrated dermal filtrates and serological titrations were carried out on the filtrates and on various components which were isolated from them by electrophoretic separa-

tion.[10] It was found that the whole dermal filtrate contained four electrophoretically distinct components which are indicated by the "peaks" labelled I, II, III, and IV in FIGURE 2. The remaining peak, ε, which appears in all the electrophoretic patterns, is due to a concentration change of buffer salts and not to a component in the preparations. At a pH of 7.9 in a buffer of ionic strength 0.05, the fastest moving com-

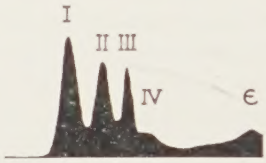

FIGURE 2. Electrophoretic pattern of concentrated dermal filtrate having L and S titers of 1:1600.

ponent, I, and the slowest moving component, IV, were present in the solution in the largest and smallest amounts, respectively. The other two components, II and III, generally appeared in about equal concentrations in dermal filtrate. On heating the preparation at 70° C. for one-half hour (which destroys the serological precipitability of L-antigen) the slower of these two middle components disappeared from the electrophoretic patterns and the concentration of the faster of these two components was increased as is shown in FIGURE 3. Components I and IV

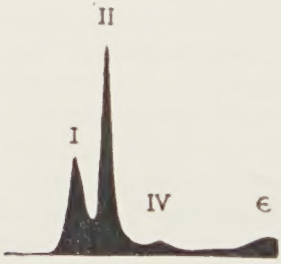

FIGURE 3. Electrophoretic pattern of dermal filtrate after heating.

were each isolated electrophoretically from concentrated filtrate and neither of these was found to precipitate in the presence of anti-vaccinal serum. The serologically active portion of the dermal filtrate was obviously associated with one or both of the middle components. Attempts to separate these components by electrophoretic means were not completely successful due to the fact that the electrical mobilities of the substances were not sufficiently different for adequate resolution. At this point in the investigation we were greatly impressed with the fact

that heating destroyed the precipitability of L and caused a disappearance of component III with an augmentation of component II. Therefore, the possibility was seriously considered that component III corresponded to L and component II corresponded to S and, furthermore, that heating transformed L to S. These ideas were subsequently abandoned.

Fractionation of dermal filtrate by precipitation at different values of pH was next undertaken.[10] Components I and IV remained in solution at pH 4.5. The material from dermal filtrate which was insoluble at pH 4.5 was only partially soluble at pH 6.5. Most of the residue could, however, be brought into solution by raising the pH to 8.6. That portion of the filtrate which was insoluble at pH 4.5 but soluble at pH 6.5 was shown to contain a single component on electrophoresis; the mobility of this substance corresponded to that of component III in whole dermal filtrate. The fraction of dermal filtrate insoluble at pH 4.5 and also 6.5 but soluble at pH 8.6 was found to consist for the most part of material with a mobility corresponding to that of component II. Reproductions of electrophoretic patterns obtained with the various fractions isolated from concentrated dermal filtrate (FIGURE 2) at different values of pH are illustrated in FIGURE 4; the results of precipitin titrations with the different solutions are also summarized in this figure.

The solution containing components I and IV was serologically inert when tested with anti-vaccinal sera. Component II, which was obtained free of component III by repeated fractionation, likewise failed to react with L- and S-antibodies. The electrically homogeneous fraction III was found to contain practically all of the L- and S-serological activity of the original dermal filtrate (FIGURE 4). A number of preparations of component III have been studied under a variety of conditions by means of electrophoresis and ultracentrifugation and in each instance the material behaved as a homogeneous substance. Since component III appeared to be a single molcular substance containing L- and S-activity its immunological properties were investigated further.

Precipitin titrations with the soluble antigens of vaccinia are regularly made by incubating the antigen-antibody mixtures in closed tubes at 50° C. for 18 hours. In view of the experiments with concentrated filtrate described above, it seemed important to eliminate the possibility that component III had only L-activity and that during incubation at 50° C. the native material was degraded to a substance which had the mobility of component II and which now for the first time was capable of precipitating with S-antibody. Serial dilutions of pure component III were set up with optimal amounts of L- and of S-antibodies and one pair of each of four such sets of titrations was incubated at 3°, 20°, 37°,

and 50° C. In each set of titrations the precipitin endpoint in the presence of S-antibody was identical with that observed in the presence of L-antibody. These findings clearly indicated that component III in its native state contained both L- and S-reacting portions.

Absorption experiments in which pure solutions of component III were treated with L-antisera showed that removal of all L-reacting material simultaneously removed all S-reacting material. Similarly, absorption with S-antibody eliminated L- as well as S-precipitinogen. The

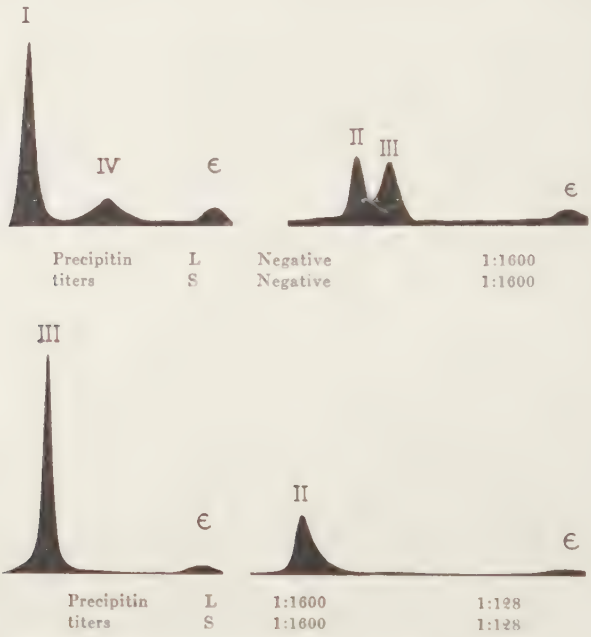

FIGURE 4. Electrophoretic patterns of fractions from dermal filtrate.

results of a typical absorption experiment are summarized in TABLE 2; they demonstrate that the two serologically distinct parts of the molecule are inseparable in their native state. This single substance possessing both L- and S-reactive parts will henceforth be called LS-antigen.

Since pure LS-antigen was now available it seemed desirable to determine whether the previously recognized degraded forms of L- and S-reacting material could be obtained by appropriate treatment of electrophoretically and ultracentrifugally homogeneous LS. This was done as follows. An unbuffered solution of LS that had a titer of 1:1600 with L- and with S-antibody was divided into two portions, one of which, A,

TABLE 2

ABSORPTION OF COMPONENT III WITH L- AND S-ANTIBODIES

Antigen	Antisera	Dilution of antigen						
		1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Component III unabsorbed	L	++++	++++	++++	++++	+++	++	+
	S	−	±	++++	++++	+++	+++	+
Component III absorbed with L-antibody	L		−	−	−	−		
	S		−	−	−	−		
Component III absorbed with S-antibody	L	−	−	−	−	−		
	S	−	−	−	−	−		

was heated at 70° C. for one-half hour, and another, B, was treated with sufficient NaOH to bring the concentration to N/20 and then heated at 56° C. for one and one-half hours, after which the pH was adjusted to 7.0. Solution A no longer precipitated with L-antibody but still had a titer of 1:1600 with S-antibody. Solution B precipitated with neither L- nor S-antibody. Inhibitory power of these preparations was investigated by adding varying amounts of solution A to constant quantities of L-antiserum, and of solution B to S-antiserum. After incubation at 50° C. for one-half hour the mixtures were tested in the usual way for precipitating antibodies. Some of the data obtained in this experiment are summarized in TABLE 3. It is apparent from these results that various levels of degradation of LS can be obtained, leaving either the L- or S-part of the molecule in a stage where it can combine with the corresponding antibody without precipitation. Since LS has been employed to represent the molecule in its native state it will be convenient to use the symbols L′S for the heat-altered antigen and L″S′ for the substance obtained after heating in the presence of alkali. In FIGURE 5 the

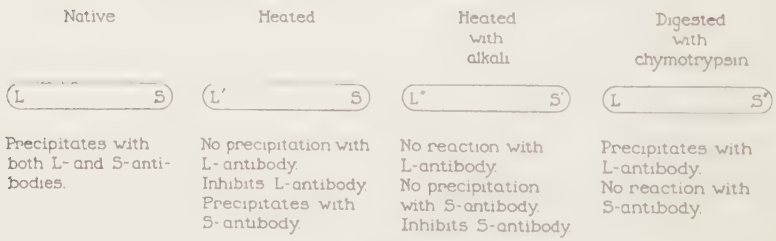

FIGURE 5.

serological activities of LS-antigen and of some of its degradation products are summarized.

Electrophoretic studies on solutions of L′S and L″S′ show that both substances are electrically homogeneous but have mobilities which differ from each other and from that of LS. Thus, in 0.05μ veronal buffer solution, pH 7.9, LS has a mobility of 4.0×10^{-5} cm./sec. per volt/cm., while L′S prepared from the same lot of antigen has a mobility of 5.9×10^{-5}. This fact is of particular interest for it clarifies an earlier perplexing observation. It is now apparent that L′S and serologically inert component II from dermal filtrate have nearly identical mobilities. L″S′ examined electrophoretically under similar conditions has a mobility

of 6.4 × 10^{-5} cm./sec. per volt/cm. The mobility of this degradation product is close to that of component I in dermal filtrate; the two are not identical, however, for the latter fails to inhibit S-antibody.

TABLE 3

INHIBITION OF L- AND S-ANTIBODIES BY DEGRADED FORM OF LS-ANTIGEN

Inhibiting solution	Ratio of inhibiting solution to antiserum	Test antiserum	Test antigen				
			1:8	1:16	1:32	1:64	1:128
None		L	++++	++++	++++	+++	++
		S	++++	++++	++++	+++	++
LS heated	1:1	L	+	−	−	−	−
LS heated with alkali	1:1	L	++	++++	+++	++	++
	0.5:1	S	−	−	−	−	−

Recently, another degradation product of LS has been obtained[11]. This substance, prepared by digesting pure LS-antigen with crystalline chymotrypsin under proper conditions, precipitates with L-antibody but not with S-antibody; furthermore, it does not inhibit the latter. This material has been designated LS″ (see FIGURE 5). It represents the degradation form of LS which was postulated on theoretical grounds in earlier reports[8, 10] to account for certain experiments in which dermal filtrate treated with S-antibody was freed of all S-precipitinogen but still contained demonstrable L-precipitinogen.

Physical and chemical properties of LS and some of its degradation products have been determined and will be reported in detail in the near future.[12] It may be briefly stated here that LS is characterized as follows: density = 1.39, specific volume = 0.72, diffusion constant = 1.5 × 10^{-7}, sedimentation constant = 4.3 s (Svedberg units), all at 4° C.; the electrophoretically determined isoelectric point is pH 4.8. Chemical analyses show 15.7 per cent nitrogen but no lipid, nucleic acid, phosphorus nor glucosamine. The LS-antigen appears to be an elongated protein molecule with a molecular weight of about 240,000 and with an axis ratio of approximately 30:1.

CONCLUSIONS

The virus of vaccinia has a complex structure since at least five antibodies develop in animals following infection or hyperimmunization with

active elementary bodies, *viz.*, a neutralizing antibody, an agglutinin designated X, antibody against a nucleoprotein constituent (NP) of the virus, and, finally, antibodies against a heat-labile (L) and heat-stable (S) soluble antigen. L- and S-antigens, although immunologically distinct, are not separate substances; they are component parts of a single substance, LS.

The LS-antigen of vaccinia provides a clear example of a single molecule which is capable of eliciting two distinct antibodies. It is theoretically possible that a third antibody, which is capable of reacting with both the L- and S-parts of the molecule, may also be elicited. However, the methods employed would not permit the identification of such an antibody.

Two levels of degradation of both the L- and of the S-parts of the LS-molecule have been demonstrated. In the first stage the property of precipitating with the corresponding antibody disappears but the power of inhibiting this antibody remains; in the next stage, serological activity is not demonstrable. It has thus been possible to prepare materials from LS-antigen which precipitate only with L- or only with S-antibody.

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PURIFICATION AND PROPERTIES OF THE PROTEIN OF THE "M SUBSTANCE" OF GROUP A HEMOLYTIC STREPTOCOCCUS*

By Charles A. Zittle and Stuart Mudd

From the University of Pennsylvania, Philadelphia.

The M substance characterizes each of the various types of virulent Group A hemolytic streptococci, as is shown by specific precipitation of extracts of the M substance with appropriate antisera.[1] The typing obtained by the use of the M substance agreed with that previously obtained for the same strains by protection tests. More recent studies[2] also have identified the M substance with the substance in intact streptococci which stimulates the production of protective antibodies in animals. The active material isolated from the neutralized N/20 HCl extracts used for typing of the streptococci has been designated the M substance by Lancefield,[3] who also showed that its reactivity with antisera is rapidly destroyed by proteolytic enzymes. Although the type-specific substance thus was shown to be a protein, it did not give rise to antibodies when injected into rabbits. Stamp and Hendry[4] modified the isolation procedure, principally by using a milder extraction temperature, and obtained a fraction which induced active immunity in mice. Heidelberger and Kendall,[5] by extracting ground streptococci with increasingly alkaline solutions, isolated a fraction which produced type-specific precipitins when injected into rabbits. Their preparations contained phosphorus. Mudd and collaborators have used sonic treatment of the streptococci and neutral extraction to obtain a fraction with type-specific properties. In a recent paper[6] summarizing these studies it was concluded that two serologically active components were present and that the predominant component was a nucleoprotein of broad reactivity and the other was a type-specific material probably related to the M substance or the T substance recently described by Lancefield.[7] In the studies of Hirst and Lancefield[2] a substance was isolated by a modification of the original method of Lancefield which induced active immunity in mice and which in rabbits gave rise to precipitins and to protective antibodies passively transferable to mice. Considerable nucleic acid was present in their preparations in addition to protein. At the same time studies done in our laboratory[8] had shown that about a third of the material extracted at 56° C. with N/20 HCl was nucleic

* The expenses of this work have been largely defrayed by a grant from The Commonwealth Fund.

acid. Our studies had also confirmed the extremely rapid inactivation produced by proteolytic enzymes and had shown that the M substance could no longer be precipitated with dilute acid from enzyme-treated solutions. We had shown further that the nucleic acid present is of the ribose type, and although the enzyme, ribonuclease, altered the acid-precipitability of the M substance it had no effect on its serological reactivity. It was concluded, therefore, that the nucleic acid did not contribute to the serological specificity of the M substance and that the active component was a protein.

In the present studies the relation of the protein and nucleic acid in the M substance has been investigated further. This work has culminated in the separation and purification of the type-specific protein. The course of this investigation and some of the properties of the type-specific protein are described. These studies were done with strains[a] 1048 and 1685 of virulent Group A hemolytic streptococci.

ISOLATION AND YIELD OF THE M SUBSTANCE[b]

Details of the isolation procedure used have been described.[8] A summary is given in TABLE 1. The amounts of the acid- and alcohol-precipitated fractions obtained have been about equal; the total of both fractions isolated has been 4.6 ± 1.3 (S. D.) per cent of the dry weight of the organisms extracted.[9] The first extracts are in most cases a golden yellow color; this is probably riboflavin as most of the color can be discharged with sodium hyposulfite. The point at which extraction is complete is strikingly demonstrated by the lack of clarity of the supernate obtained after centrifuging. While the M substance is being extracted from the organisms the supernates are clear; where extraction is complete even prolonged centrifuging does not give a clear supernate and the organisms do not pack as well. The small amount of material sometimes obtained in these turbid extracts is non-specific in its precipitation with antisera.

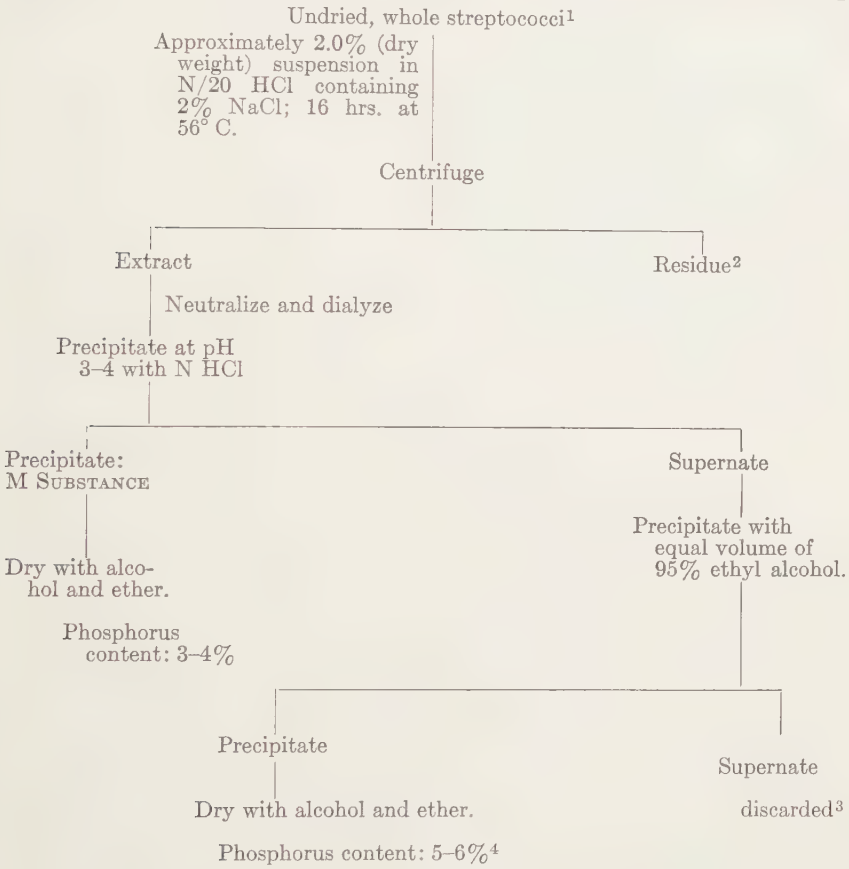
The acid-precipitated fraction, on the basis of phosphorus content

[a] These strains in previous reports were designated Types 6 and 1 respectively and had so been typed in Dr. F. Griffith's laboratory.[9] Recent studies by Dr. Rebecca C. Lancefield and Dr. Alice C. Evans with strain 1048 have shown that it is not Type 6. There is no question of the virulence and the group classification of this strain but at present its type has not been definitely assigned. Reference to dry cultures prepared when the strain was received in this laboratory have led to the same conclusion. Dr. Lancefield has assigned the 1685 strain to Type 3. Dr. Griffith in reporting the type of this strain commented that in addition to colonies of Type 1, colonies were obtained which reacted with both Type 1 and 3 antisera. He had not yet decided whether the reaction with Type 3 antiserum was a group reaction. This does not entirely explain Dr. Lancefield's finding but it may be that in this strain both Type 1 and 3 antigens are present as Lancefield[10] has found for strain C203 although perhaps to a different or variable degree.

[b] The term, M substance, is retained for the material obtained from neutralized and dialyzed HCl-extracts by precipitation with acid, for historical reasons and also because to some degree it does represent a definite compound of protein and nucleic acid in salt-like union. The purified, active, protein component of the M substance is designated the M-protein.

TABLE 1

ISOLATION OF THE TYPE-SPECIFIC M SUBSTANCE FROM THE GROUP A HEMOLYTIC STREPTOCOCCI



[1] The organisms were grown 18 hours by a routine procedure which yielded about 0.2 gm. of dried streptococci per liter of culture medium. Recently, under the direction of A. M. Pappenheimer, Jr., the cultures have been kept neutral by the addition of NaOH during growth and yields of more than 1.0 gm. per liter obtained. **Bernheimer, A. W., & Pappenheimer, A. M., Jr.** Jour. Bact. In press. The yield of the M substance per unit weight of dry bacteria has been about the same from organisms grown in this manner as from those grown by the previous method.

[2] The Group A specific polysaccharide has been regularly isolated by us from this dried residue by Fuller's hot formamide method. The yields of polysaccharide have been about the same as with intact streptococci.

[3] Negligible amounts of material remain in this supernate. In Lancefield's original method for preparing extracts of streptococci, the Group A specific polysaccharide was isolated from this supernate. Her extractions, however, were made in a boiling water bath. Our extracts at 56° yielded only minute amounts of the group-specific polysaccharide.

[4] This fraction also contains M-protein. The ratio of protein to nucleic acid is about the reverse of that in the M substance. This is fractionated further with acid and alcohol into 3-4 per cent and 5-6 per cent phosphorus fractions. The former is essentially M substance and is added to it; the latter which is largely ribose nucleic acid, will be purified by methods suitable for nucleic acid. Considerable of this nucleic acid will dialyze through cellophane and some must have been lost in the preparatory dialysis.

(3–4 per cent), is about two-thirds protein and one-third nucleic acid, whereas in the alcohol-precipitated fraction 5-6 per cent phosphorus this proportion is reversed. On this basis about 2.5 per cent of protein has been obtained from the streptococcus. Some part of this is not the M-protein as judged by the appearance of variable amounts of insoluble material in the purification. However, it appears that this strain of the streptococcus contains about 2 per cent of the M-protein.

Although reprecipitation of a particular preparation of the M substance with acid at the point of optimum flocculation gave phosphorus values close to the original, precipitation of the same lot at two different pH values gave different phosphorus contents, with less at the higher pH. These results suggested that a considerable amount of the nucleic acid was free and not firmly bound to the protein.[c] This was confirmed by the electrophoretic data to be discussed later.

SEPARATION OF THE M-PROTEIN

The protein is precipitated from solutions of the M substance by half saturation with ammonium sulfate made neutral with ammonium hydroxide. After being chilled, the precipitate is removed by centrifuging, and is dissolved in a small amount of water and dialyzed against 3 to 6 successive 2 liter portions of distilled water in the refrigerator for 24 to 48 hours. Some of the protein precipitates during the dialysis. Precipitation is completed by the careful addition of N. 10 HCl. When the nucleic acid content has been reduced to about 1.0 per cent, maximum precipitation occurs at pH 5.0. With larger amounts of nucleic acid, the pH for maximum precipitation is at lower values. In routine practice one volume of 95 per cent ethyl alcohol is added before adjustment of the pH, since in this case the point of maximum precipitation is much more striking.

The supernate from the ammonium sulfate precipitate is dialyzed and then precipitated by the addition of several volumes of 95 per cent ethyl alcohol and adjustment of the pH. This fraction, designated "nucleic acid" in TABLE 2, still contains some M-protein.

The data obtained in numerous experiments with ammonium sulfate

[c] When the extractions were performed with the stirring produced by convection currents obtained by partly immersing the vessel used in the 56° bath, 2 to 3 extractions were required to obtain all of the M substance. By the use of thorough mechanical stirring all of the M substance can be obtained in one 16-hour extraction period; in many cases, one 6-hour extraction has been sufficient.

[d] Although the nucleic acid appears to be free, on the basis of chemical fractionation and electrophoresis, polar forces no doubt exist between the protein and nucleic acid and precipitates of the two represent a salt-like combination. The term, protein nucleate, will be used to describe such combinations. It will be seen that the M substance can be regarded as a protein nucleate. The term nucleoprotein is used to designate compounds of nucleic acid and protein with non-polar bonds such that electrophoresis and chemical fractionation will not separate them. The plant viruses are nucleoproteins in this sense.

are given in TABLE 2. A complete separation of protein and nucleic acid is not obtained in one precipitation. In successive precipitations the nucleic acid content of the protein fraction is reduced about 60 per cent each time. The small amount of nucleic acid remaining after the third precipitation does not represent a minimum. Further precipitations were not done because the preparations were satisfactory for the other

TABLE 2

SUMMARY OF PROCEDURE FOR SEPARATING M-PROTEIN AND NUCLEIC ACID BY PRECIPITATING WITH AMMONIUM SULFATE; DISTRIBUTION AND ANALYTICAL DATA FOR VARIOUS FRACTIONS

Procedure	Fraction (characteristic)	Recovery		Phosphorus content, %[3]
		%	% original[1]	
1. Combined original *acid* precipitated preparations.			100	3–4
2. Solution and reprecipitation with acid and alcohol.	Insoluble[2]	5	5	—
	Protein	65	65*	3.0
	Nucleic acid	20	20	6.5
3. Solution and precipitation of protein fraction of 2 with ammonium sulfate; recovery of precipitate and supernate.	Insoluble[2]	4	2.5	—
	Protein	40	26 *	1.0
	Nucleic acid	40	26	4.5
4. Repetition of 3 with protein fraction of 3.	Insoluble[2]	4	1	—
	Protein	70	18 *	0.3
	Nucleic acid	15	4	1.8
5. Repetition of 3 with protein fraction of 4.	Protein	85	15	0.15
	Nucleic acid	10	2	1.0

[1] The figures marked * in this column represent the portions that are reprecipitated. Addition of the other parts gives the amount recovered—75.5 per cent. The amount unaccounted for is largely nucleic acid lost by dialysis. In a number of cases, the original acid-precipitated preparations were combined, ground in a mortar for easier solution, and step 3—ammonium sulfate precipitation—was carried out at once. In this case, recoveries had been even lower (in a summary of averaged data, 69 per cent). Analysis of the data showed the loss was in the high-phosphorus fraction corresponding to the "nucleic acid" of step 2, for which dialysis was not used. The dialyzability of the nucleic acid was shown by comparative data for weight recovery and phosphorus recovery. In every case, the latter was smaller, in several instances 60 per cent as compared to 80 per cent.

[2] The appearance of insoluble material with successive drying and solution should not be regarded as evidence of alteration of the M-protein due to drying with alcohol and ether, since in numerous fractionations no insoluble material appeared after the solution of the combined original preparations. Some of our best preparations have withstood frequent drying with alcohol and ether without obvious changes in solubility. In fact, our experience suggested that any preparation which would not dissolve after such drying to give a 1-2 per cent solution in N/10 salt at neutrality was not pure. Another characteristic of a pure preparation is lack of color. This will be discussed later.

[3] The nucleic acid content can be obtained by multiplying these values by 10.5. It had previously been shown[8] that the phosphorus represents nucleic acid.

work to be described. The final yield of M-protein after repeated precipitations is 15 per cent of the starting material. This yield is increased when the M-protein in the "nucleic acid" fractions is recovered by similar repeated precipitation.

Preparations of the M-protein obtained by this procedure contained 14.4 per cent nitrogen after drying in the vacuum oven at 50° C. No correction was made for the ash content which probably was small because prolonged dialysis was used. Several preparations were homogeneous by electrophoresis and one preparation studied was homogeneous by sedimentation and diffusion.

OTHER METHODS TRIED FOR THE SEPARATION OF PROTEIN AND NUCLEIC ACID

Precipitation of the nucleic acid in the M substance with barium was tried with no success. A small amount of precipitate was obtained but subsequent isolation of the protein revealed it still contained considerable nucleic acid.

Another method tried for the separation of the protein and nucleic acid was precipitation with glacial acetic acid. It was hoped this would be successful with the high nucleic acid-fractions. High and low nucleic acid-fractions were obtained by this means but the separation of protein and nucleic acid was inefficient.

Ammonium sulfate precipitation at neutrality was used with the nucleic acid, alcohol-precipitated fractions (see TABLE 1) but was not practical because of the large loss of nucleic acid during dialysis. In several experiments only 50 per cent of the starting material was recovered. In parallel experiments with yeast, more than 80 per cent of nucleic acid was recovered. This suggests that the molecular weight of the nucleic acid in the streptococcal extracts was considerably less than the 17,000 estimated by Loring[11] for yeast nucleic acid. It seems unlikely that the streptococcal nucleic acid was homogeneous in size because smaller amounts were lost with successive dialysis of this material.

The nucleic acid content of the M substance could be reduced by warming the latter with N 100 NaOH, followed by neutralization, dialysis and recovery of the protein. This method was not used extensively because considerable loss of protein occurred.

INCREASE IN SOLUBILITY OF THE M-PROTEIN AT ELEVATED TEMPERATURE

In working with an aqueous, 0.2 per cent solution of the M-protein it was observed that the flocculent precipitate obtained at pH 5.2 at room

temperature could be dissolved by warming at 56° C. On cooling this solution, the M-protein reprecipitated. Subsequently, it was found that neutral solutions of appropriate concentration would show this phenomenon also, *i.e.*, the solubility of the protein, either isoelectric or as a salt, was considerably enhanced by an increase in temperature. When warm saturated solutions of the M-protein were cooled the appearance of the precipitate differed markedly from that obtained by isoelectric or salt precipitation. The precipitate was apparently granular although it did not settle rapidly. Examination of this material in the dark field showed the presence of small spherical particles about 5 microns in diameter. When crushed they were seen to be solid but apparently soft. This may represent a stage of crystallization. However, repeated precipitation by this method has failed to give definite crystals. All preparations of the M-protein examined have behaved similarly.

ELECTROPHORETIC EXPERIMENTS

This part of the work has been carried out in collaboration with Dr. Florence Seibert of the Henry Phipps Institute.[12] The apparatus and technic developed by Tiselius[13] were used. Data obtained with the M substance are given in TABLE 3. In four of six preparations examined

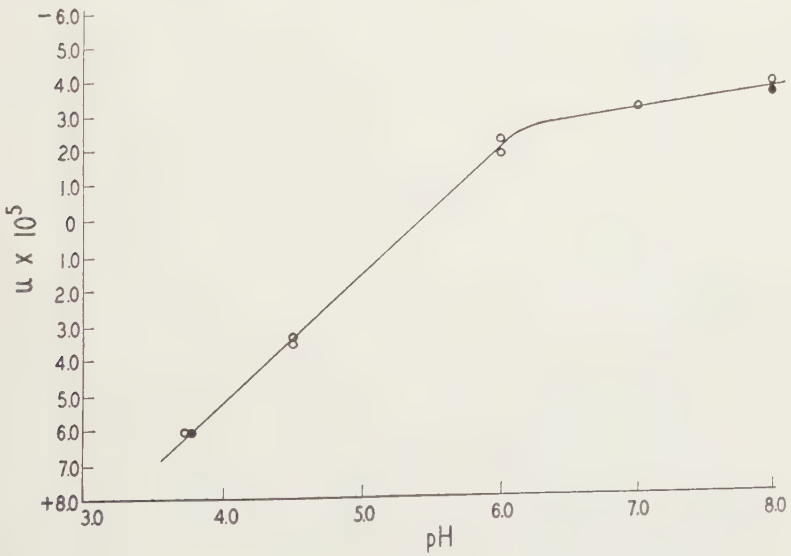


FIGURE 1. Mobility of the M-protein in acetate (pH 3.7 and 4.5) and phosphate (pH 6.0, 7.0 and 8.0) buffers, μ 0.1. The mobility (u) is in terms of cm.^{2} volt^{-1} sec.$^{-1}$.

only the two components mentioned were observed; in the other two a small amount of a third component with a mobility of −6.0 at pH 6 appeared. In several experiments the components described in the table were isolated. The results obtained with the preparation containing 2.3 per cent phosphorus shown first in the table are typical. The material isolated from the cell to which the fast component had migrated contained 7.7 per cent phosphorus. Thus, the fast component was identified with the nucleic acid. The material from the cell containing the slow component contained 1.1 per cent phosphorus and reacted type-specifically with homologous antisera. Subsequent work showed that the nucleic acid contained in the material in this cell was due to incomplete separation. Curves showing the mobility of the purified M-protein at various pH values are presented in FIGURE 1. The open circles represent preparations of the strain 1048 M-protein and the solid circles preparations of the strain 1085 M-protein. No difference was observed between the proteins of the two types studies. Also there was little or no difference in the mobility of the protein when purified or when admixed with nucleic acid as in the M substance.

TABLE 3

MOBILITY OF THE PROTEIN AND NUCLEIC ACID IN PREPARATIONS OF THE M SUBSTANCE

Preparation	Phosphorus content	Concentration, total, by weight	pH Phosphorus buffer (μ 0.1)	Mobility (u) x 10^5 cm.^{2} volt^{-1} sec.$^{-1}$, [1-4]	
				Protein	Nucleic Acid
Standard procedure except precipitation at pH 4.7.	2.3%	0.6%	6.0	−2.3	−11.4
Standard procedure and reprecipitation.[5]	1.1	0.5	6.0	−2.2	−10.3
Standard procedure and reprecipitation.	1.1	0.5	8.0	−3.8	−14.2
Prepared from organisms grown 4 hours.	3.4	0.25	8.0	−3.6	−14.3

[1] The current for these runs was 13.4 to 13.8 milliamperes, the potential gradient 5.85 to 7.12 volts cm.$^{-1}$. Part of the variation in the latter was due to the use of cells of different cross section.

[2] The mobilities given are for the descending boundary which has been shown to give the truer mobility, uncomplicated by ionic concentration effects.

[3] The sign designates the charge on the molecule, *i.e.*, a negative sign indicates movement toward the positive pole.

[4] Throughout the text, mobilities are given as whole numbers; the dimensions are as indicated here.

[5] In the course of several reprecipitations, this preparation was warmed with N/10 NaOH and treated with ribonuclease[3] which probably assisted in reducing the nucleic acid content.

Electrophoretic examinations were made of other streptococcal fractions because of their possible relation to the M-protein. Some representative observations are given in TABLE 4.

Another protein-nucleic acid fraction (NPA)[14] that we have examined is obtained by neutral extraction of streptococci disintegrated with sonic vibrations.[6] In some respects, it appears to be similar to the "neutral-extracted nucleoprotein" described by Heidelberger and Kendall.[5] The material prepared as described[6] contains variable amounts of nucleic acid. Yields of 15 to 25 per cent of the dry weight of the streptococci have been obtained. The mobilities of the protein when present in the NPA and of the protein when isolated from the NPA and purified were the same and the serological properties of the two were similar. The mobility of the NPA-protein was considerably greater than that of the M-protein. Serological data suggested the presence of about 1 per cent of type-specific substance in NPA.[6] However, no M-protein was observed electrophoretically in any of the NPA preparations studied. It was also absent from an original sonic extract examined in 1.5 per cent concentration.

Some data are shown for purified streptococcal nucleic acids.[e] The mobilities of the nucleic acids are of similar magnitude to those observed in the mixtures of nucleic acid and protein as isolated. The values were somewhat lower than obtained in two experiments with yeast nucleic acid (-15 at pH 6.0, -19 at pH 8.0). Stenhagen and Teorell[15] have reported mobilities for thymus nucleic acid of -17.4 at pH 5.86 and -20 at pH 7.90 and μ 0.1.

The mobilities obtained for the vitreous humor polysaccharide are the same for the two pH values at which it was studied. A similar lack of change in mobility with change in pH has been reported for heparin.[17] The streptococcal capsular polysaccharide with chemical composition similar to the vitreous humor polysaccharide[18, 19] would be expected to behave similarly. No such component was observed in any of the streptococcal preparations examined.

The group-specific polysaccharide had negligible mobility and probably belongs to the group of "neutral" polysaccharides.[20] It was separated by electrophoresis from one of the nucleic acid preparations[16] and its identity confirmed by electrophoretic examination of material isolated by Fuller's formamide method[21, 22] from the acid-extracted residue (see TABLE 1).

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[e] These nucleic acids were prepared by Dr. M. G. Sevag and Mr. J. Smolens.[14]

TABLE 4

ELECTROPHORETIC DATA FOR OTHER STREPTOCOCCAL COMPONENTS

Preparation	Concentration, total by weight	Phosphorus content	Phosphorus buffer μ 0.1, pH	Mobility (u) x 10^5 cm.^{2} volt^{-1} sec.$^{-1}$		
				Protein	Nucleic acid	Other components
NPA	0.7%	3.6%	6.0	−6.2	−11.7	−5.0; small amount
Principal protein component of NPA (P_2)[4]	0.2	<0.1	6.0	−5.8		
NPA	0.9	1.5	8.0	−7.1	−16.3	−14; visible only on ascending side
Principal protein component of NPA (P_2)[4]	1.0	<0.1	8.0	−7.7		Immobile boundary (both sides), separated by compensation device—no ppt., with anti-serum
Nucleic acid (N_2) from NPA[4]	1.0	9.10[1]	6.0		−12.3	Immobile boundary separated, no activity with antisera
Nucleic acid (MZR) from sonic residue[4]	1.0	8.87[2]	6.0		−13.2	Immobile boundary separated, reacted with Group A streptococcus antisera. Separated nucleic acid reacted only with pneumococcus antisera.[16]
Nucleic acid (N_2) from NPA[4]	0.5	9.10[1]	8.0		−17.1	
Vitreous humor polysaccharide[3]	0.5		6.0			−10.4
Vitreous humor polysaccharide[3]	0.5		8.0			−10.3
Streptococcus Group A specific polysaccharide	1.0		7.0 (μ 0.02)			−0.2

[1] Contained 25 per cent thymus-type nucleic acid (diphenylamine reagent); the remainder presumably was yeast-type nucleic acid since positive color tests for yeast-type nucleic acid were obtained. This preparation was shown to be pure nucleic acid by its containing the theoretical amount of phosphorus and the equivalence of the phosphorus and purine nitrogen values.

[2] This preparation contained no thymus-type nucleic acid.

[3] This material, prepared from bull's eyes, was studied because the streptococcal capsular polysaccharide has been shown to be similar chemically.[18, 19]

[4] Prepared by Dr. M. G. Sevag and Mr. J. Smolens.[14]

SEDIMENTATION AND DIFFUSION BEHAVIOR OF THE M-PROTEIN

These experiments were performed by Dr. A. M. Pappenheimer, Jr.[23] in the laboratory of Physical Chemistry at the University of Wisconsin. Several sedimentation velocity experiments were performed using a standard Svedberg oil-turbine centrifuge from which a sedimentation constant (S_{20}) of 1.7×10^{-13} cm./sec./dyne was calculated. The scale average displacement curves obtained were still sharp at the end of these experiments (2.5 and 4.5 hours, at 60,000 r.p.m.) indicating that little diffusion had taken place and suggesting a high degree of dissymmetry for the sedimenting unit. No other components were observed by sedimentation and this homogeneity was confirmed by diffusion. A diffusion constant (D_{20}) of 4.2×10^{-7} cm.^{2}/sec. was calculated from two diffusion experiments. With these constants and value of 0.77 for the partial specific volume, the molecular weight was calculated to be approximately 41,000. The frictional coefficient ratio (f/f_0) calculated from these data was 2.2, which indicates an extremely elongated molecule with a ratio of major to minor axis of at least 20 to 1. This large axial ratio was confirmed by viscosity measurements. In neutral M/10 sodium phosphate at 22.5° C. the relative viscosity of a 1.00 per cent solution was 1.35, of a 2.00 per cent solution 2.00. The reciprocals of these values when plotted against protein concentration fall on a straight line through the origin. Treffers[24] recently showed the validity of this relation for numerous proteins. The axial ratio calculated from the viscosity data with Polson's[25] empirical equation relating viscosity and axial ratio was of the same magnitude as that given above.

SEROLOGICAL PROPERTIES OF THE PURIFIED M-PROTEIN

The chemical work described in the previous sections was done with material isolated from two strains of streptococci, representing two different types: strain 1048, and strain 1685. Larger amounts of material were available from strain 1048 because of the better growth of this strain. In many cases preliminary work was done with the M preparations of this strain but in all of the chemical work parallel experiments were eventually done with material from the 1685 strain, and in no case was any significant difference observed.

In the precipitin test the 1685 M-protein gives considerable cross-reaction with 1048 streptococcal antisera. The M-proteins used in these tests were homogeneous by electrophoresis. When the M-protein is

treated with trypsin, the precipitation with heterologous antisera is lost just as rapidly as the type-specific precipitation. These observations are strong evidence for the reality of this cross-reaction because they show that contamination is unlikely and that the cross-reaction has the same lability to trypsin as the specific reaction. The nature of this cross-reaction was brought out by quantitative determinations of the amount of precipitate obtained at several dilutions. These data are presented graphically in FIGURE 2.[f] The milligrams of N shown are the amounts precipitated from 1.0 milliliter of antiserum. FIGURE 2 curve I gives

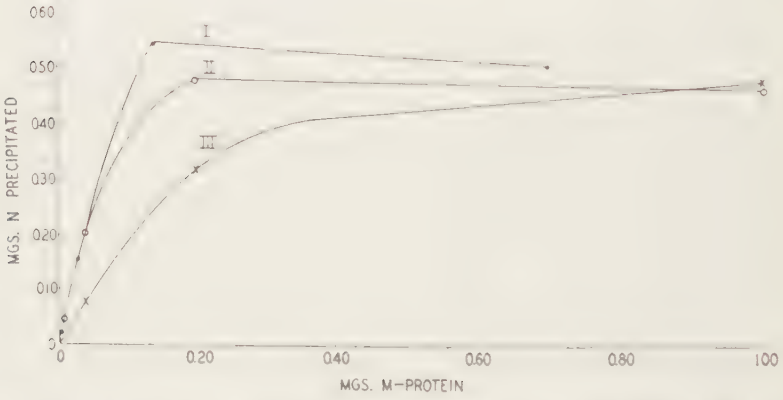

FIGURE 2. The precipitation of streptococcal antiserum 1.0 ml. with homologous I and II and heterologous (III) M-protein.

data for the precipitation of a preparation of the 1048 M substance with 1048 antiserum and curve II precipitation of the purified 1048 M-protein with the same serum. The curve for the M substance is on the basis of its M-protein content. In curve III the precipitation of the 1685 M-protein with the 1048 serum is shown. All of these precipitations were performed with the same lot of pooled serum. The 1048 homologous antibodies of this serum could be completely removed by using an excess (1 mg. to 1 cc. of serum) of the heterologous 1685 M-protein. Neither the purified 1048 M-protein nor the 1048 M substance reacted with a group-specific antiserum[g] that reacted with dilutions of 1:2,000,000 of the purified group-specific polysaccharide. Hence both of these preparations must contain less than 0.1 per cent of the polysaccharide and

[f] The point of inflection of curve I was confirmed by testing the supernates from the precipitates with antigen and antibody. The true point of inflection of curve II is slightly to the left of the point shown, since similar tests revealed a slight excess of antigen at this point.

[g] This antiserum was furnished by the Lederle Laboratories.

the presence of this polysaccharide can not explain the slightly larger amount of precipitate obtained with the M substance.

To complete the serological studies, experiments were performed in which preparations of the purified M-protein were used to precipitate streptococcal antisera that protected mice against relatively large doses of homologous streptococci.[h] The precipitates were removed and the protective power of the antisera tested again. These experiments were done in parallel. Dilute solutions of the 1048 M-protein (0.2 mg. in 1.0 ml. of 0.85 per cent NaCl was used to precipitate 1.0 ml. of antiserum, see curve II of FIGURE 2 , were effective in removing the protective antibodies from antisera. An antiserum which, in 0.5 ml. amounts, protected a mouse against 10 minimal lethal doses (MLD) of streptococci, would no longer protect against 1 MLD.

Similar experiments made with the 1685 M-protein were not so successful, although a partial absorption of the protective antibody was obtained. The 1685 M-protein preparation used was not highly purified (it still contained about 10 per cent nucleic acid) and had not been studied in the electrophoresis apparatus. However, it represented a stage in the purification of a lot that in electrophoresis was a single substance and serologically it was a representative preparation, *i.e.*, it gave the cross reaction described with the 1048 antiserum and typical behavior with homologous 1685 antiserum. The preparation was type-specific (type 3) in its reactivity with absorbed antisera[i]. It may be that the rigorous treatment used in its purification, such as drying with alcohol and ether, etc., had partially denatured it so that some reactive groups remained but not its full potency as an absorbing antigen. The passive protection experiments were essentially specific although in a few experiments some cross-protection was obtained between the 1685 and 1048 strains. We have observed in active protection tests cross-protection between these same strains in that life was prolonged, but survival was not obtained as in the homologous test. These observations are in line with the cross reaction observed in the precipitin reaction (see FIGURE 2.).

DISCUSSION

The method of purification of the M-protein was designed to free it primarily of nucleic acid and secondarily of the small amount of other

[h] These studies will be published in detail elsewhere. They are part of a program of related studies being performed by Miss Maria Wiener. We are grateful for the use of the results of these studies at this time.

[i] We are very grateful to Dr. Rebecca C. Lancefield for the absorbed, type-specific antisera and "M extracts" for precipitin tests for comparison with our own materials. Her generous cooperation and interest in these studies is greatly appreciated.

protein which occasionally appears to be present. Preliminary tests have shown that the M-protein and the NPA-protein, which is the most likely contaminant of the former, are precipitated by about the same salt concentrations. Therefore, further purification by this means would be impossible or very difficult. Fortunately, extraction of the streptococci with dilute acid has considerable selectivity and only rarely was contamination other than nucleic acid observed in the M substance. The purified M-protein preparations, with one exception, have not shown any other definite component in electrophoresis although occasionally in electrophoretic observations made by the Svensson slit optical method the advancing limb of the concentration gradient-curve was unsymmetrical with respect to the other limb, suggesting the presence of small amounts of another component. The single exceptional preparation of the M-protein contained almost an equal amount of another component with the same mobility as the NPA-protein. In ten other M preparations examined no similar large contamination had been seen. This does illustrate, however, the need for a specific purification method. The observed increase in the solubility of the M-protein at higher temperature and subsequent precipitation on cooling seems to offer the desired specificity. Work in progress has shown already that the dark brown color accompanying some of the preparations can be eliminated by this means.

Some of the properties previously described for the M substance are of interest in view of the knowledge that it is a protein nucleate.[d] One of these interesting properties is its inability to precipitate with acids after a very brief treatment with proteolytic enzymes. One can conclude that brief digestion of the protein has produced units so small and soluble that nucleic acid will not precipitate them. A number of other proteins have been tested with nucleic acid before and after proteolytic digestion but in no case has a similar complete loss of precipitation been observed.

The form in which the M-protein occurs in the streptococcus is of great interest. The following evidence strongly supports the conclusion that it is very likely in a form different from that which we have described: (a) Little or nothing can be extracted from the streptococci by neutral solvents, whereas the M-protein isolated from the acid-extracts is quite soluble in neutral solvents. (b) The M-protein has been clearly related by absorption experiments to the protective antibodies produced with streptococcal vaccines. However, the M-protein produces very little protection when injected into animals. The protection obtained, although definite, is much less than that given by streptococci containing an equivalent amount of M-protein. (c) The physical data obtained for

the M-protein which show the ratio of major to minor axis is about 20 to 1 suggest, in the light of our knowledge of other proteins, that the protein as prepared may be in denatured form. If this is a denatured protein it has some properties usually not found in the denatured form, namely, its considerable solubility and also its excellent reactivity with antibodies, presumably to the native form of the M-protein since they were obtained with intact streptococci. However, it may represent a denatured protein or a part of a larger antigenic unit. The evidence presented in (a) and (b) would be compatible with the latter hypothesis. The appearance of nucleic acid in the acid-extracts that otherwise appear to be specific for the M-protein suggest a probable association of these two substances in the streptococcus.

We have been impressed with the apparent specificity of dilute acid in the extraction of the M-protein and its concomitant nucleic acid. To summarize, (a) electrophoretic examination of the M substance has shown it to be almost exclusively M-protein and nucleic acid; (b) the total yield of M substance is about the same whether extraction is carried out at 56° or 37° C.; (c) negligible amounts of the group polysaccharide are found in the acid extract of whole streptococcal cells whereas treatment of the acid-extracted residue by Fuller's method has yielded the same amount of this polysaccharide that exists in intact streptococci; (d) the nucleic acid in these acid extracts is always of the ribose type. The desoxyribose type is present in the streptococci also and has been extracted after sonic disintegration of the cells.

All of this suggests a specific extraction by acid of a particular part of the streptococci, perhaps a component of the cell-wall[26, 27] or part of it. We have other evidence also for the localization of the M-protein. Sonic treatment makes possible solution of 15 to 25 per cent of the streptococci by neutral solvents but only negligible amounts of the M-protein are present in solutions obtained by this procedure. This suggests that perhaps the M-protein is a part of the cell-wall which is disrupted but not brought into solution by this treatment. What seems to be direct evidence for this is obtained in electron microscope observations of sonically treated streptococci.[26] "Shadow" cells are seen which result from the loss of considerable electron absorbing material, probably the cell contents, since the size and shape of the cell are retained. The evidence we have given suggests a localization on the cell-wall. Other evidence suggests it is probably only a part of the bacterial cell-wall. Dr. Lancefield, investigating the action of trypsin on living streptococci, has observed that streptococci in cultures treated with trypsin remained viable but attempts to isolate the M substance from them

were unsuccessful.[28] This evidence and the studies of streptococcal variants strongly suggest that the M-protein, although a part of the cell-wall, may be highly specialized, having a protective role against phagocytosis[1] but not being otherwise necessary for the viability of the streptococcus.

[1] Virulent strains of streptococci have been shown to be less readily phagocyted than non-virulent variants of the same strain; combination of components of the bacterial surfaces with homologous antibodies renders the bacteria susceptible to phagocytosis.[9]

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COMPLEMENT*

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INTRODUCTION

Complementary activity has been studied for more than forty years but complement has defied all attempts at isolation.[1] Its properties are only partially known and its structure has not been determined. Indeed, Brocq-Rousseu and Roussel[2] recently stated that "complementary power comprises all the activities of unheated serum."

Complement is usually defined as the non-specific portion of fresh serum which is not increased by immunization, and which when added to sensitized cells results in their destruction. In other words, *complement is still defined in terms of its function and not in terms of its constitution.*

The power of complement to hemolyze sensitized red blood corpuscles is only one of its many properties. Among others are: (1) the lysis of certain sensitized bacteria;[3] (2) the opsonization of certain bacteria;[4] (3) the activation of thermostable immune opsonins;[5] (4) the acceleration or augmentation of the aggregation of certain antigens by their homologous antisera, whether the antigen is a bacterial suspension,[6] a protein in solution,[7] or a suspension of erythrocytes;[8] and (5) the capacity to kill bacteria (bactericidal action) in the absence of bacteriolysis.[9]

It has been claimed that there is evidence showing that (6) a thermostable constituent of complement operates in the destruction of viruses by immune sera.[10] It has been postulated that (7) a toxin-antitoxin complex binds complement *in vivo*,[11] and that by this mechanism the toxin is neutralized. Furthermore, (8) complement has been said to be associated with the coagulation processes of blood,[12, 13] but recent evidence appears to invalidate this contention.[14, 15, 16] (9) There seems to be a relationship between the sedimentation rate of erythrocytes and complementary activity,[17] *i.e.*, a fast sedimentation rate was found to be related to a low complementary activity. (10) Complement has even been claimed to be associated with muscle contraction.[18]

It should be pointed out that complement, because of its multiple components, may be found to promote one of the aforementioned functions while failing to promote others; *e.g.*, a deficiency of one or more of

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the components of complement may prevent hemolysis without affecting phagocytosis. Therefore, any activity of complement is determined by the substrate upon which it operates; and it thus becomes necessary in defining complementary activity or titer to define also the substrate employed. In this paper, unless otherwise noted, the substrate referred to is sheep red corpuscles sensitized with rabbit anti-sheep red cell serum.

At various times it has been suggested that complement is a simple chemical agent like oleic acid,[19] a lypolytic enzyme,[20] a proteolytic enzyme,[21] a catalyst,[22] a peptidase,[23] or more commonly a physicochemical state or a collodial attribute of fresh serum.[1] These theories, however, do not explain the fact that complement can be separated into two or more functionally distinct components, each of which is essential for the exhibition of its activity.

GENERAL PROPERTIES OF COMPLEMENT

Complement deteriorates rapidly with age. It loses its activity in a few days at 0° C., in a few hours at room temperature, and in a few minutes at 56° C. Hydrogen-ion concentrations acid to a pH of 5 and alkaline to a pH of 9 readily inactivate complement.[24] The activity of complement is inhibited by both hypotonic and hypertonic salt solutions, and especially by divalent ions.[25, 26] It is destroyed by shaking,[27] ultraviolet light[28] and proteolytic enzymes.[29] The optimum activity of complement occurs at a pH of 7.2–7.4 in physiological salt solution (0.9 per cent NaCl). Complement may be preserved for months by the process of freezing, drying and storing *in vacuo*.[30]

The narrow range of conditions under which complement is stable has contributed largely to the failure of earlier attempts at isolation.

PAST KNOWLEDGE CONCERNING THE COMPOSITION OF COMPLEMENT

It is known that treatment of serum with distilled water,[31] carbon dioxide[32] or dilute HCl[33] separates it into two thermolabile fractions which individually have no complementary activity; when combined, almost full activity is restored. The soluble fraction has been designated as the "albumin fraction" or the "end-piece," while the water-insoluble fraction has been termed the "globulin fraction" or the "mid-piece."

It has also been shown that yeast inactivates a relatively heat-stable component of complement, namely the "third component;"[34] while dilute ammonia destroys another fraction known as the "fourth com-

TABLE 1

RESUMÉ OF PAST KNOWLEDGE CONCERNING THE CONSTITUTION OF COMPLEMENT

COMPLEMENT			
Treatment with yeast cells	Dilution and acidification		Treatment with dilute ammonia
Removal of third component	Precipitate	Supernatant	Removal of fourth component
The third component is:	"Globulin fraction" or "mid-piece" is:	"Albumin fraction" or "end-piece" is:	The fourth component is:
1. Destroyed at 66° C. for 30 minutes;	1. Destroyed at 56° C. for 15 minutes;	1. Destroyed at 56° C. for 15 minutes;	1. Destroyed at 66° C. for 30 minutes;
2. Destroyed by cobra venom.	2. Unstable at room temperature.	2. Stable in ice-box for at least 48 hours.	2. Associated with bound serum calcium (?);
			3. Associated with lipids (?).

ponent."[35] Claims have been made for the occurrence of other components, but definite evidence of the existence of these is lacking.[1, 36] A schematic diagram of the components and their properties is given in TABLE 1.

THE ASSAY OF HEMOLYTIC COMPLEMENT

Three methods are commonly used in the quantitative estimation of complement in hemolysis:

1. The serial tube method is most widely employed. This method determines the smallest amount of serum necessary to cause complete hemolysis of a standard amount of sensitized red blood cells. The reciprocal of this minimum quantity is then assumed to be the complement titer in units.

Unless all procedures are controlled by careful standardization of the concentration of erythrocytes, by accurate estimation of the potency of the hemolysin employed, regulation of time and temperature, elimination of the effect of naturally occurring antibodies, correction for possible experimental variations and the degree of resistance of different red blood cells to hemolysis, serious errors may obscure otherwise significant results.

2. An alternative method which is often used measures the degree of hemolysis produced by a known amount of complement acting on a constant volume of red cells until the action is complete. This is known as the method of 50 per cent hemolysis.[37, 38] However, the same meticulous care must be employed in this method as in the serial tube method.

3. The observation that the complementary action of a given serum may be taken as a coefficient of the time required to produce complete hemolysis has led to the employment of another method.[39] The length of time needed to hemolyze a constant volume of sensitized corpuscles is assumed to be inversely proportional to the titer or activity of the complement.[40]

From the numerous criticisms which have been levelled at the available methods of complement titration[1] it is obvious that improvements are desirable. The present authors have availed themselves of the observation that the smallest amount of serum necessary to initiate hemolysis of a standard unit of red cells may be taken as a basis of comparison.[41] As most of the experiments detailed below involve the use of this method, the exact procedure employed in this laboratory is given.

Brooks' physiologically balanced salt solution, or in some instances when a buffered salt solution is undesirable, 0.9 per cent saline solution, is the diluent employed. This is previously boiled for one hour and

brought up to volume. The erythrocytes are obtained daily from healthy sheep from which the blood is drawn under aseptic conditions. The blood of the same animal is used throughout the course of any given experiment. One volume of cells is washed four times in ten volumes of either Brooks' solution or 0.9 per cent saline, and standardized to about 500,000,000 corpuscles per cc. Generally the cells are prepared just before the experiment, and are kept not longer than 12 hours so that errors due to increased cell fragility are avoided. Five units of a rabbit anti-sheep hemolysin of a titer of at least 1:4000 are used to sensitize the cells. After mixture of cells and hemolysin, the sensitized cells are allowed to stand at room temperature for about 15 minutes prior to use. The final mixture contains 250,000,000 erythrocytes per cc.

The blood to be tested for complementary activity is allowed to clot, and the clear serum immediately separated by centrifugation, care being taken to avoid hemolysis of the red blood cells. In no instance should this take over two hours from the time of bleeding. The serum is immediately diluted with the salt solution, 1:30 in the case of guinea pig serum, and 1:15 in the case of human serum; after dilution the serum is immediately titrated.

A series of 15 serological test tubes (acid-cleaned and freed of the last traces of acid with doubly-distilled water) is set up, and the diluted complement is carefully added in amounts ranging from 0.01 to 0.15 cc. with a 0.1 cc. pipette graduated in hundredths. Salt solution is then added to each tube so as to make a final volume of 0.15 cc. in each tube. One cc. of the standardized and sensitized cells is then added to each tube with rotation of the tube. The tubes are then well shaken by hand, and incubation is carried out in a water bath at 37° C. for 30 minutes. The tubes are then centrifuged at 1800 r.p.m. for 5 minutes; and the tube showing the first sign of hemolysis (usually a yellow-orange color, *not red*) is taken as the initial point of hemolysis. With the normal guinea pig serum this is seen at about 0.02 cc. of a 1:30 serum dilution, while in human serum it generally occurs at 0.02-0.03 cc. of a 1:15 serum dilution. A control tube, containing only one cc. of the standardized cells plus 0.15 cc. of salt solution, should be carried through as above, so as to avoid errors indicative of cell fragility. The advantages of this method are obvious. Cell degradation products capable of inhibiting the function of complement are avoided. The use of an excess of amboceptor eliminates the error incurred by the natural hemolysin (anti-sheep hemolysin). Only a small amount of serum is needed for this test; and finally, the complement is allowed to operate according to its concentration,

By the use of this method, and avoiding all variations of procedure, results are obtained with an accuracy of ± 5 per cent.

THE PREPARATION, SPECIFIC INACTIVATION, AND REACTIVATION OF THE COMPONENTS OF COMPLEMENT

An improved method for the separation of the mid-piece and end-piece by the carbon dioxide method follows. By the use of this procedure little or no denaturation occurs in either of these two components.

Distilled water is saturated at room temperature with pure carbon dioxide gas which is allowed to bubble through the water for 30 minutes under slight pressure. One cc. of serum is added to 9 cc. of the carbon dioxide-saturated water, and carbon dioxide gas is again passed through the mixture for 20 minutes. Foaming produced by the bubbling of the gas is reduced by the addition of capryl alcohol with a wooden applicator stick dipped in the alcohol. Care should be taken to avoid an excess of alcohol because of its hemolytic quality. The globulin precipitated after 20 minutes saturation of the serum with carbon dioxide is rapidly centrifuged in an angle-centrifuge at room temperature at a speed of 4000 r.p.m., and resuspended three times in distilled water, after each time again being centrifuged as above. The euglobulin precipitated in the fractionation of one cc. of serum is then redissolved in 10 cc. of saline, and the residual carbon dioxide is taken off *in vacuo*.

The end-piece, which is present in the supernatant after the precipitation of the mid-piece, is made isotonic with 17 per cent NaCl solution, and the carbon dioxide is removed *in vacuo*. Both fractions are then tested for neutrality with brom-thymol blue. Each fraction should be inactive by itself, but almost fully active when combined with the other.

Although dilute ammonia is commonly used for the specific inactivation of the fourth component, it has been the practice in this laboratory to use hydrazine[42] instead of ammonia. It has been shown that at a pH of over 9 the mid-piece and the third compenent are unstable.[43] Furthermore, it was found that hydrazine inactivates the fourth component without any ill-effects on the other components. Therefore, to inactivate the fourth component of complement specifically, 0.15 cc. of 0.16 M hydrazine is added to one cc. of serum, thoroughly mixed and incubated in the water bath at 37° C. for one hour. The pH of such a mixture is 7.2-7.4. After incubation it is diluted with 9 cc. of 0.9 per cent NaCl solution.

Fresh yeast, or a yeast-powder (zymin), is usually employed to remove or inactivate the third component specifically. However, several workers have reported that the end-piece and the mid-piece are also

destroyed by yeast or zymin.[44] In this laboratory the third component is usually inactivated by an insoluble carbohydrate recently isolated from fresh yeast.[45] With this agent, *only* the third component is removed, and no inhibition due to diffusible substances occurs. The method is as follows: 10 to 15 mg. of the insoluble carbohydrate is boiled for 30 minutes in 10 cc. of 0.9 per cent saline, centrifugalized, and the supernatant decanted. One cc. of serum is added to the carbohydrate sediment, shaken, and incubated for two hours. The mixture is further shaken every 30 minutes. After the two hour incubation 9 cc. of 0.9 per cent saline is added, the contents mixed, and then centrifugalized until clear. The supernatant is decanted, and contains all the fractions of complement with the exception of the third component.

As to the procedure of reactivation, it is the practice here to employ quantities of the components or inactivated complements identical with the smallest amount of the untreated serum needed to cause *complete* hemolysis of the standard unit of red blood corpuscles employed. For reactivations the initial point method of hemolysis is not employed because more concentrated serum components are needed and these in turn interfere with the reading of initial hemolysis.

The inactivated complements or components are combined, incubated at 37° C. in the water bath for 15 minutes, and the solutions titrated for complementary activity. The complementary titer is compared with that of an equal amount of the untreated serum and this is usually expressed in percentage of the original titer of the serum complement.

NEWER KNOWLEDGE OF THE COMPONENTS OF COMPLEMENT

All attempts to isolate complement or its components have hitherto failed.[1, 44] Several investigators have tried to fractionate whole complement and its components with neutral salts such as ammonium sulfate.[46, 47, 48] The results are discordant and too indefinite to characterize any one of the components as a functionally distinct entity. Other methods, such as adsorption, extraction, and fixation have also proved to be fruitless.

It became apparent that although a great deal is known regarding the general properties of whole complement, little or nothing has been learned concerning the properties of its individual components. Therefore it is clear that before isolations are attempted, the optimum conditions for their stability and activity should be ascertained. Studies in this direction were made and the results of these experiments follow.

VARIATIONS IN GUINEA PIG COMPLEMENT

Because of its high content of hemolytic complement guinea pig serum was employed in all of the experiments described below. However, guinea pigs show wide seasonal variation in the complementary activity of their sera.

Several investigators[46, 50, 51] stated that complement suffers a reduction in titer during the winter months, and they attributed the seasonal variation to a deficiency in green foods and to a lack of vitamin C. Again, certain investigators[52] attempted to discover whether or not vitamin C entered into the constitution of complement since ascorbic acid does not exist in a free state in the blood. They claim that it is bound to proteins or lipoids but no proof is given for this statement. They found that the complementary power of the serum of rabbit was increased by the administration of vitamin C, and that a proportionately low value of vitamin C occurred in the adrenals of guinea pigs in which low complement titers were found.[52, 53] Later it was shown that a correlation exists between the complement titer and ascorbic acid content of guinea pig serum.[41] A similar qualitative relationship was also shown to occur in man.[54, 55, 56,§] Since these findings were made, it has been the practice in this laboratory to maintain all guinea pigs on a well-balanced diet supplemented with at least 10 mg. of ascorbic acid daily.[57]

It has also been noted that the action of whole native complement is associated with a redox mechanism.[57] The nature of the effect of vitamin C *in vivo* and of the redox mechanisms *in vitro* are suggestive, but as yet there is no explanation for either phenomenon. It would appear that only the third component is implicated in these phenomena. Also it is the opinion here that the vitamin C saturation is an index of an animal's well-being, and that the observed increase in complement in guinea pigs on high vitamin C diets is indicative of either the availability or the state of those serum entities responsible for complementary activity.

THE EFFECT OF VARIOUS HYDROGEN ION CONCENTRATIONS ON THE COMPONENTS OF COMPLEMENT

Although it is generally agreed that a pH under 5 and over 9 inactivates complement, nothing was known concerning the effect of pH

§ **Crandon, Lund & Dill,** (New England Jour. Med. **223**: 353. 1940), in a controlled study of vitamin C deficiency in a human reported that no relationship existed between the ascorbic acid content and complementary titer of blood plasma. However, the late date (78 days after onset of deficiency) on which the complement titrations were started and the possible technical differences of methods employed may account for this result. A larger series of cases with strict conformance to standardized technique would be desirable.

on the individual components of complement. The present authors[43] were able to show that end-piece and the fourth component are relatively unstable in acid solutions and stable in alkaline solutions; and that the mid-piece and the third component of complement are relatively unstable in alkaline solutions and more stable in acid solutions. This is illustrated in TABLE 2. It is noted that after treatment of serum with 0.1 N NaOH to a pH of 9.5 the selective reactivation is brought about only by ammonia-treated serum and the intact mid-piece. This indicates that both the third component and the mid-piece are inactivated at this alkaline pH. On the other hand, the treatment of serum with 0.1 N HCl to a pH of 4.2 resulted in its selective reactivation by zymin-treated serum and the intact end-piece. Therefore, acids attack the fourth component and the end-piece. These observations aided greatly in the final separation of the components of complement from whole serum as is discussed subsequently.

THE EFFECT OF AMINO COMPOUNDS ON THE COMPONENTS OF COMPLEMENT

Although it was demonstrated in 1926 that dilute ammonia destroys the fourth component of complement,[35] the nature of this inactivation was not explained. It has been postulated that the inactivating process is due to the formation of an un-ionized calcium-ammonium double salt.[35] Attempts have also been made to associate this inactivation with lipids and bound calcium.[55, 59] The present authors in collaboration with Dr. J. Seifter[42] formulated the hypothesis that the ammonia-inactivation of the fourth component is the conversion of the active carbonyl groups of the component to some less active structure. To test this hypothesis, various amino compounds of two general classes were tested for their anti-complementary effects: those amino compounds which are known to react with carbonyl groups, and those which do not. The results are summarized in TABLE 3. It is noted that (1) only primary amines, hydrazine, phenyl hydrazine and α-methyl hydroxylamine specifically inactivate the fourth component; (2) simple alkalinity of amines is not the cause of the inactivating property; (3) the fourth component is inactivated only by amino compounds that are known to have decided reactions with aldehydes; (4) the $-NH_2$ group is necessary for this inactivation; (5) the type of substituent group replacing a hydrogen atom of ammonia to produce the amino compound has a pronounced influence on the capacity of the compound to inactivate the fourth component; (6) amino compounds which have been previously treated with aldehydes do not affect the fourth component; and (7) polar groups

TABLE 2

THE EFFECT OF VARIOUS HYDROGEN ION CONCENTRATIONS ON THE COMPONENTS OF COMPLEMENT

Complement treated with:	pH*	Reactivations with:							
		Per cent hemolysis**	Heated serum	NH_4OH serum	Zymin serum	Mid-piece	End-piece	Heated mid-piece	Heated end-piece
		Percentile restoration of complementary activity							
NaOH (0.1 N)	10.4	0	0	0	0	0	0	0	0
NaOH (0.1 N)	10.0	0	0	0	0	10	0	0	0
NaOH (0.1 N)	9.5	0	15	100	0	100	5	15	0
NaOH (0.1 N)	9.2	10	60	100	20	100	15	30	10
NaOH (0.1 N)	8.8	60	100	100	100	100	60	90	60
NaOH (0.1 N)	8.4	90							
Untreated	7.1	100							
HCl (0.1 N)	6.1	90							
HCl (0.1 N)	4.9	40	90	60	100	40	100	40	80
HCl (0.1 N)	4.5	10	30	15	85	10	100	10	20
HCl (0.1 N)	4.2	0	20	0	75	0	60	0	0
HCl (0.1 N)	4.0	0	0	5	0	0	0	0	0
HCl (0.1 N)	3.5	0	0	0	0	0	0	0	0

* Adjusted to 7.2 before titration.
** Titer of original serum taken as 100 per cent.

TABLE 3*

THE EFFECT OF AMINO COMPOUNDS ON THE FOURTH COMPONENT OF COMPLEMENT

Substituent type	Reagent	Formula	Minimal molarity	Maximal molarity	pH	Inactivation of fourth component
Positive	Ammonia	$H–NH_2$	0.16		8.5–10	+
	Methylamine	$CH_3–NH_2$	0.12	0.24	7.4– 8.0	+
	Dimethylamine	$(CH_3)_2$ NH		0.72	7.0–10	–
	Trimethylamine	$(CH_3)_3–N$		0.72	7.0–10	–
	Tetramethyl ammonium hydroxide	$(CH_3)_4–NOH$		0.96	7.0–10	–
	Ethylamine	$CH_3CH_2–NH_2$	0.12	0.24	8.0– 9.0	+
	Diethylamine	$(CH_3CH_2)_2–NH$		0.72	7.0–10	–
	Triethylamine	$(CH_3CH_2)_3–N$		0.72	7.0–10	–
	Hydrazine	$H_2N–NH_2$	0.08		7.2– 8.0	++
Negative	Phenylhydrazine	$C_6H_5NH–NH_2$	0.24	0.36	7.0– 7.4	+
	Aniline	$C_6H_5–NH_2$		0.64	7.0–10	–
Polar	Urea	$NH_2CO–NH_2$		0.95	7.0–10	–
	Acetamide	$CH_3.CO\ NH_2$				
	Glycine	$COOH.CH_2–NH_2$		0.96	7.0–10	–
	Semicarbazide	$H_2N.CO.NH\ NH_2$		0.72	6.0–10	–
	Thiosemicarbazide	$H_2N.CS.NH–NH_2$		0.72	7.0–10	–
	Hydroxylamine	$HO–NH_2$		0.72	6.0–10	–
Blocked polar	α-methyl hydroxylamine	$CH_3.O–NH_2$	0.16	0.24	8.5– 9.5	+
Indifferent	Methenamine	$(CH_2)_6–N$		0.96	7.0–10	–

* Taken from **Pillemer, L., Seifter, J., & Ecker, E. E.** Jour. Immunol. **40**: 89. 1941.

which potentially lend acidic or redox properties to amino compounds completely abolish the anti-complementary effect of the amine. Blocking of the polar group restores this activity, as in the case of α-methyl hydroxylamine. In addition, amines which have negative and polar groups apparently need extra-physiological conditions of pH and temperature in order for the $-NH_2$ group to interact with aldehydes.

These results suggest that the active amino compounds act upon a carbohydrate complex of the end-piece, and that the end-piece acts as a carrier for the fourth component-carbohydrate complex.

These findings may also explain the past failures to purify complement by fractionation with ammonium sulfate because, if care is not taken to maintain a low temperature and a slightly acid pH, inactivation of complement readily occurs.

THE EFFECT OF SALT CONCENTRATION, TEMPERATURE AND DIALYSIS ON COMPLEMENT AND ITS COMPONENTS

Dialysis of whole serum against distilled water separates complement into a "globulin fraction" and an "albumin fraction."[60] The "globulin fraction" is termed "mid-piece." Mid-piece is unstable in 0.9 per cent saline.[60] In fact, it loses its activity in a few hours. However, it remains active and stable if suspended in distilled water[60, 61] or hypertonic salt solution.[62] It is inactivated by heating at 54° C. for 30 minutes,[63] and is non-dialyzable against water, hypotonic or hypertonic salt solutions.[64, 65]

The "albumin fraction" or "end-piece" is relatively stable on standing. At 2° C. its activity remains unimpaired for several days.[60, 66] It is also inactivated by heating at 54° C. for 30 minutes,[63] instead of at 56° C. for 30 minutes as had been previously reported. It is also non-dialyzable against water, hypotonic or hypertonic salt solutions.[64, 65]

The fourth component has a stability of the same order as the end-piece.[42] It is non-dialyzable,[65] but has a higher thermal inactivation, a temperature of 65° C. for 30 minutes being required to inactivate it completely.[63] In 10 per cent NaCl the thermostability of the fourth component is reduced to 61° C. for 30 minutes.

The third component is very unstable, and is the first to disappear in serum on standing at room temperature or on ice.[1, 65] It is non-dialyzable,[65] and is inactivated at a temperature of 62° C. for 30 minutes.[63] In 10 per cent NaCl the inactivation temperature is reduced to 60° C. for 30 minutes. Hypertonic salt solutions have a protective action on the removal of the third component from whole serum by yeast, zymin or the insoluble carbohydrate of yeast.[67]

THE ROLE OF CALCIUM AND THE EFFECT OF LIPID SOLVENTS

Calcium

It has been generally assumed that serum calcium is associated with complement function, although differences of opinion exist as to the nature and action of the calcium. Some authors state that the removal of the diffusible calcium does not influence complementary activity;[35] but others claim that ionized calcium is necessary.[68]

The present authors investigated the role of calcium in complement constitution and function.[43] It was found that, whenever calcium was removed from an acid medium, a certain degree of acid inactivation took place; but the removal of calcium up to 98 per cent from a neutral or slightly alkaline medium had no effect on any component of complement. Calcium therefore plays a role of doubtful significance in complement function.

The results of these experiments are given in TABLE 4. It is noted that calcium dis-ionizing organic acids inactivate complement in a manner similar to the hydrogen-ion effect of HCl (see TABLE 2). However, their alkali salts, in amounts up to 5 per cent, had no effect on

TABLE 4‡

THE EFFECT OF CALCIUM DISIONIZING ORGANIC ACIDS AND THEIR ALKALI SALTS, OF SODIUM HEXAMETAPHOSPHATE AND LEAD PHOSPHATE ON COMPLEMENT.

Complement treated with:	pH*	Per cent hemolysis†	Reactivations with: Heated serum	NH_4OH serum	Zymin serum	End-piece	Mid-piece	Heated end-piece	Heated mid-piece
			Percentile restoration of complementary activity						
NH_4OH	7.2	0	90	0	100	100	10	85	0
Citric acid	4.9	0	25	0	80	100	10	15	0
Lactic acid	4.8	0	25	0	80	100	10	10	0
Tartaric acid	4.9	0	25	0	90	100	5	10	0
Oxalic acid	4.9	0	20	0	100	100	5	10	0
$Pb_3(PO_4)_2$	7.2	0	0	80	0	0	60	0	0
5% $Na_2(Na_4P_6O_6)$	6.8	100							
5% alkali citrates, oxalates and tartrates	7.2	100							

* Adjusted to 7.2 before titration.
† Titer of original serum taken as 100 per cent.
‡ This table is taken from **Pillemer, L., & Ecker, E. E.** Jour. Immunol. **40**: 101. 1941.

complement. It is also noted that sodium hexametaphosphate, which places the calcium so effectively in the anion complex that it is no longer available for ordinary analysis, likewise had no anti-complementary effect. Lead phosphate adsorbs 98 per cent of the serum calcium, as well as the mid-piece, but there is no effect on the fourth component which supposedly contains calcium.

These results show that no definite relationship exists between calcium and the fourth component. In fact, the weight of evidence indicates that it is not involved in the immediate labile and reversible reactions of complement. It is the belief of the authors that calcium may be a structural part of the end-piece - fourth component complex, but not essential to its function, because, when acids are used to remove the calcium, the removal is accomplished at the expense of protein denaturation and the destruction of end-piece structure. The relationship of calcium to complement as observed by other investigators then becomes an index of the degree of complement denaturation.

Lipid Solvents

It is known that the treatment of serum with ether,[68] followed by the removal of the ether, inactivates complement. It was later reported that the factor destroyed by ether or chloroform was the fourth component.[58] As benzene inactivated a supposedly new fraction of complement, a hypothetical "fifth component" was postulated, but its presence was not confirmed.[59]

The present authors showed that the extraction in the cold of active dehydrated complement with absolute alcohol, anhydrous ether, or petroleum ether does not inactivate complement.[69] In fact, the extracted lipids were anti-complementary, and complement often showed an increase of activity after such extractions. As stated above, no evidence of a possible "fifth component" was found.

In addition,[36] it has been shown that treatment of fresh serum with ether, chloroform, cadmium chloride, benzene or benzine had no specific effect on any one component, but inactivated fresh complement by virtue of protein denaturation. While it is agreed that the phospholipids may play a role in complementary function, it has not as yet been possible to establish a definite relationship.

THE ACTIVE ANTI-COMPLEMENTARY PRINCIPLE IN FRESH YEAST

Although the inactivation of complement by yeast was described as early as 1900,[34] the nature of this inactivation remained obscure.

The present authors[45] isolated an insoluble fraction from fresh yeast which is composed of 94 per cent carbohydrate and is insoluble in hot water, organic solvents and cold alkali. The yield was about 2 per cent.

This insoluble carbohydrate fraction inactivated specifically the third component of complement in an amount of only one twenty-fifth of the required amount of fresh yeast. None of the soluble fractions from yeast inactivated the third component. Since the anti-complementary factor is undoubtedly an insoluble complex carbohydrate, the inactivation of third component appears to be due to the adsorption of this relatively heat resistant component of blood serum.

EARLY ATTEMPTS AT THE PURIFICATION OF COMPLEMENT

Adsorption

As early as 1906 it was shown that complement is removed from serum by coagulated serum proteins.[70] Since that time many attempts have been made to adsorb complement on various adsorbing agents. Berkefeld filter, kaolin, alumina, carbon, insoluble calcium and magnesium compounds, barium sulfate, starch, inulin, agar, gelatin, and even bacteria have been used for this purpose.

While it has been suggested that the adsorbents remove only the mid-piece,[12] most investigators agree that no selective adsorption of any component occurs.[1, 71]

In view of the successes obtained in the purification of enzymes by adsorption and elution, controlled adsorption was undertaken in attempts to isolate or purify complement.[67] However, elution of the adsorbed complement and its components was found to be impossible, but certain observations were made in the course of these experiments and they are as follows: (1) Lead phosphate and titanium oxide remove the mid-piece of complement. (2) Kaolin and magnesium hydroxide are less specific in their action. (3) Aluminum hydroxide gel removes the total complement complex. (4) A correlation exists between mid-piece and third component and the amount of euglobulin phosphorus adsorbed.

Fractionation with Ammonium Sulfate

Although serious attempts have been made to separate complement from whole serum, the results have been too indefinite to allow the characterization of either complement or any of its components as functionally distinct entities. Also, past failures to purify complement can be attributed to lack of care in avoiding denaturation of the labile complement.

However, it was recently shown[72] that the addition of one volume of guinea pig serum to 14 volumes of 2.4 M ammonium sulfate results in the precipitation of about 35 per cent of the total serum proteins and that this precipitate contains, after the removal of the ammonium sulfate, about 90 per cent of the complementary activity originally present in the serum. Nevertheless, attempts to separate this material into the various components of complement were futile. It then became evident that, although the 1:15 dilution of the serum with ammonium sulfate solution sufficed to separate the globulins from serum without much loss of complementary activity, the same method was unsatisfactory for further purification of the complement components.

In FIGURE 1 is presented a fractional precipitation curve of the guinea pig serum globulins.* It is noted that there are four distinct salting-out plateaus. As to the complement component content of these fractions, the following were noted: (1) The material precipitating between 1.40 M and 1.56 M ammonium sulfate had a small amount of mid-piece activity, but was very unstable. (2) The proteins precipitating between 1.56 M and 2.2 M ammonium sulfate contained varying amounts of third com-

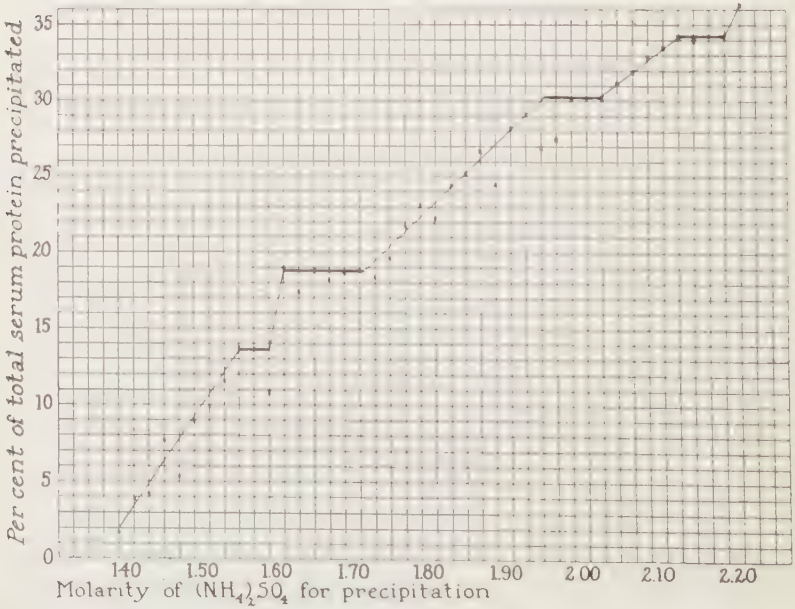

FIGURE 1. Fractional serum protein precipitation curve. Serum proteins precipitated from 1:15 serum at 2° C. by various concentrations of ammonium sulfate.

* Unpublished experiments performed by C. B. Jones and S. Seifter in collaboration with the authors.

ponent, fourth component and end-piece. However, the results were not clear-cut and characterization of any of these fractions proved to be impossible.

THE SEPARATION AND CHARACTERIZATION OF THE COMPONENTS OF COMPLEMENT

With the collaboration of Prof. E. J. Cohn and Dr. J. L. Oncley of Harvard University, the present authors have been able to separate three of the components of complement in a high degree of purity and have characterized these components physico-chemically and immunologically.[65] This was accomplished with exact physico-chemical manipulations.

To guide the work described below, electrophoretic diagrams were prepared of mid- and end-piece (made by the carbon dioxide method) as well as of zymin- and ammonia-treated sera.[73] The results indicate that the so-called "globulin fraction" or mid-piece contains at least four distinct proteins, two of which have electrophoretic mobilities faster than those originally present in whole serum; while the so-called "albumin fraction" or end-piece also contains at least four distinct proteins, one of which appears to carry nearly all of the γ-globulin originally present in whole serum. No significant difference was detected electrophoretically between normal serum and serum deprived of its fourth component by treatment with ammonia. Serum deprived of its third component by treatment with zymin showed a disturbance of the α-globulins, accompanied by a slight increase of the mobilities of the remaining serum proteins. It is evident, therefore, that the terms "mid-piece," "end-piece," "albumin fraction," and "globulin fraction" are unsatisfactory and misleading as applied here. Therefore, after discussions and agreement with Dr. M. Heidelberger of Columbia University, the components of complement have been designated as C′1, C′2, C′3 and C′4,[73] corresponding to the mid-piece, end-piece, third component, and fourth component respectively in the older terminology.

However, to avoid confusion, in this paper the older terminology is used in designating the components, but the suggested terminology will be followed in all subsequent publications from this laboratory.

Since the components involved in complement for hemolysis appear to be associated with serum proteins, it seemed important to isolate them in the highest degree of purity obtainable and to characterize them. This was successfully accomplished with three of the components of complement, *i.e.*, mid-piece, end-piece, and fourth component. The separation was achieved under rigidly controlled conditions by fractional precipitation with ammonium sulfate, accompanied by various extrac-

tions and dialysis procedures. The method is published in detail elsewhere.[65]

Mid-piece was found to be a euglobulin with an apparent iso-electric point of 5.2; with an electrophoretic mobility of 2.9×10^{-5} in phosphate buffer of ionic strength 0.2 at pH 7.7; and with a sedimentation constant of 6.4×10^{-13} in KCl of ionic strength 0.2. Its activity was destroyed by heating at 50° C. for 30 minutes, by hydrogen ion concentrations alkaline to its iso-electric point, and also in dilute solutions. It exhibited a definite inhibitory effect in protein concentrations exceeding 0.02 per cent, while full activity was observed at protein concentrations between 0.002 and 0.02 per cent. It comprised 0.60 per cent of the total serum proteins.

The end-piece and fourth component were present in the same serum fraction. The final purified protein was a muco-euglobulin, 98 per cent of which had an electrophoretic mobility of 4.2×10^{-5}. The final yield was 0.18 per cent of the total serum proteins. Its apparent iso-electric point was about 6.3-6.4. It contained 10.3 per cent carbohydrate, and had a specific optical rotation of $-192.5°$. Immunologically, this serum fraction appeared to play a dual role. Treatment at 50° C. for 30 minutes destroyed all of the end-piece activity, while the fourth component was totally destroyed only by heating at 66° C. for 30 minutes. The fraction therefore performed two immunological functions, one by virtue of a heat-labile constituent, and the other by virtue of a relatively heat-stable constitutent.

The properties of these purified fractions are contrasted in TABLE 5. It is noted that the apparent iso-electric point, that is, the pH of water triturated with the precipitated euglobulins associated with end-piece and fourth component, actually was close to 6.3, while that of the mid-piece was 5.2. The fraction with the higher mobility and precipitated by the higher concentration of salt thus had the more nearly neutral iso-electric point. A more detailed study of this unexpected result will be undertaken in order to examine the interactions of these components of complement with one another, and with the other components of serum.

Third component was found in small quantities in nearly every fraction of serum. Attempts at the purification of this component by specific adsorption with the insoluble carbohydrate from fresh yeast[45] and subsequent elution are now in progress.

The three purified complement components (mid-piece, end-piece and fourth component) comprise about 0.8 per cent of the total serum proteins. Recently,[74] employing an entirely different technique (fixation) it has been found that one cc. of guinea pig serum contains from 0.15 to

0.2 mg. of complement protein. However, in a personal communication to the authors, Heidelberger now reports that he finds 0.4 to 0.7 mg. of complement protein to be a safer approximation. This is in good agreement with the values found by fractional precipitation. The variability of the amount of complement fixed by an antigen-antibody complex may be due to the relative amounts of each component fixed. Work now in progress in this laboratory points to such a possibility.

TABLE 5*

CHARACTERIZATION OF THE MID-PIECE AND THE END-PIECE AND THE FOURTH COMPONENT OF COMPLEMENT

	Euglobulin (mid-piece)	Muco-euglobulin (end-piece and fourth component)
Electrophoretic mobility x 10^5 pH 7.7; ionic strength 0.20	2.9	4.2
$S=\frac{1\%}{20^\circ,w} \times 10^{13}$ In potassium chloride of ionic strength 0.20	6.4	
Per cent protein nitrogen	16.3	14.2
Per cent total serum protein	0.72	0.17
Per cent carbohydrate	2.7	10.3
Per cent phosphorus	<0.1	<0.1
$[\alpha]$ 25° C.	$-28.7°$	$-192.5°$
Apparent iso-electric point	5.2–5.4	6.3–6.4
Percent original complementary activity	100	85
Heat stability	Destroyed at 50° C. for 30 minutes.	End-piece destroyed at 50° C. for 30 minutes; fourth component at 66° C. for 30 minutes.

* This table is taken from **Pillemer, L., Ecker, E. E., Oncley, J. L., & Cohn, E. J.** Jour. Exp. Med **74**: 297. 1941.

CONCLUSIONS

It is now established that three of the components of complement are distinct chemical entities. Therefore, past contentions that surface tension or collodial states alone can explain complementary activity are no longer tenable. In fact, the complementary activity of serum may

now be viewed as the result of interactions between chemical entities, *and the destruction or removal of any of these substances alters the activity of the complement complex.*

Mention should be made that in the treatment of bacterial diseases, actively or passively, it has been assumed that complement is always present in abundance. Although it is now known that three of the complement substances comprise only about 0.8 per cent of the total serum proteins, it is evident that this is not a safe assumption, in that these factors, in infections, may be depleted faster than they are produced. They may therefore be of greater diagnostic value, and may even prove to possess prognostic and therapeutic significance.

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THE QUANTITATIVE RELATIONSHIP BETWEEN ANTIGEN AND ANTIBODY IN THE PRECIPITIN REACTION

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Heidelberger and his co-workers, by the application of quantitative methods to the study of the precipitin reaction, have shown that for many systems the relationship between antibody and antigen in the precipitate formed when antigen is added to an excess of antibody can be expressed by the equation,

$$y = 2Rx - \frac{R^2x^2}{A}$$

where y = mg. antibody precipitated.
x = mg. antigen added.
A = Total antibody present.
R = Ratio $\frac{\text{Antibody}}{\text{Antigen}}$ at the equivalence point.

Although good agreement is obtained between the values calculated from this equation and those found experimentally for many systems which have been carefully studied,[1] in other systems certain changes must be made in the equation in order to obtain agreement.[1]

In 1935 it was shown by Heidelberger and Kendall[1a] that this equation could be derived by an application of the law of mass action if certain assumptions were made. The authors were aware that the assumptions upon which this derivation was based were over simplified. Therefore, although the derived relationship explained the two most striking *quantitative* aspects of the precipitin reaction, namely, the changing ratio between the two components in the precipitate and the lack of a pronounced volume effect, it is necessary to reexamine the theoretical basis upon which it rests.

The following assumptions form the basis of a modified theory. Some of the assumptions rest upon independent evidence so substantial that they must be accepted, and others are logical conclusions drawn from available information concerning the properties of antigens and antibodies.[2] The assumptions are:

1. Antibodies are modified serum proteins.
2. The reaction between antigen and antibody is between specific groups or areas upon the surface of the molecules.

3. Both antigen and antibody may be multivalent with respect to each other, *i.e.*, each molecule may possess more than one reactive group or area. The reactive groups upon a given molecule may all have the same specificity or they may be different. The maximum number of molecules of antibody bound by one antigen molecule may be determined by the number of reactive groups upon the antigen or it may be limited by steric factors. There is a limit to the number of antibody molecules that can be arranged about an antigen molecule of finite size.
4. Within the limits imposed by steric factors the reactivity of any specific group upon the antigen or antibody molecule is independent of the state of combination of other specific groups upon the same molecule. The reactivity of an antigen or antibody group upon the surface of a precipitate is the same as the reactivity of the same group on the surface of a molecule in solution.
5. An equilibrium is established between the free and combined antigen and antibody groups in the system. It is evident that the system under consideration is not homogeneous. It is concerned with the reaction of groups upon larger surfaces and the equilibria established will be between reactive groups upon the surface of precipitates and the molecules in solution.

Inasmuch as both antigens and antibodies are complex protein molecules which may vary widely in their properties a satisfactory theory must allow for these variations.

Thus, antigens may vary in molecular weight and in the number and kind of reactive groups upon their surfaces. In some cases all of the reactive groups may react as if they had the same specificity, whereas in others part of the groups may react with separate antibodies. The reactive groups may be arranged upon the antigen in such a way that they react independently of each other. They may also be arranged in such a way that the reaction of one group prevents the reaction of other groups.

The antibody may also show the same type of variations. The antiserum produced by immunizing an animal with any antigen may contain a mixture of antibody molecules which differ in the number and kind of reactive groups and in the firmness with which they combine with the antigen.

Because of the complex possibilities of antigen-antibody systems the discussion of the theoretical consequences of the assumptions made will be divided into a number of sections. The theory will be developed first for the simpler cases and then extended to the more complicated systems.

In the first case to be considered, it is assumed that the reaction is irreversible and that all of the reactive groups upon the antigen and all the antibody molecules in the antiserum are alike.

If the valence of antibody is 1, in the presence of an excess of antibody all of the reactive groups upon the antigen will be combined with whole antibody molecules. The relationship between A, the number of antibody molecules combined, and B, the number of antigen molecules added, would be

$$A = NB \text{ where } N = \text{valence of antigen,}$$

$$\text{or, } \textit{Mg. Antibody pptd.} = rN \times \textit{mg. antigen added,}$$

where r is the ratio between the molecular weights of antibody and antigen.

If the valence of antibody is 2, in the presence of an excess of antibody all of the antigen groups will be in combination, but some of the antibody molecules will have both groups free, some will have one group combined, and some will have both groups combined. If there is a perfectly random distribution of the available antigen groups between the reactive groups upon the antibody, the most probable number of molecules of antibody combined in the different ways can be computed.

Let

A = Number of molecules of antibody in the system.

B = Number of molecules of antigen added.

N = Valence of antigen.

NB = Number of antigen groups added, and also = Number of antibody groups combined.

$\frac{NB}{2A}$ = Fraction of antibody groups combined and also the probability that any particular antibody group is combined.

$\frac{2A - NB}{2A}$ = Probability that any particular antibody group is free.

$\left(\frac{2A - NB}{2A}\right)^2$ = Probability that any antibody molecule has both groups free.

$\left(\frac{NB}{2A}\right)^2$ = Probability that any antibody molecule has both groups combined.

$2\left(\frac{2A - NB}{2A}\right)\left(\frac{NB}{2A}\right)$ = Probability that any antibody molecule has one group free and one combined.

The number of uncombined antibody molecules would then be

$$\left(\frac{2A - NB}{2A}\right)^2 A = A - NB + \frac{\overline{NB}^2}{4A}.$$

The number of singly combined antibody molecules would be

$$2\left(\frac{2A - NB}{2A}\right)\left(\frac{NB}{2A}\right)A = NB - \frac{\overline{NB}^2}{2A}.$$

The number of doubly combined antibody molecules would be

$$\left(\frac{NB}{2A}\right)^2 A = \frac{\overline{NB}^2}{4A}.$$

The total number of combined antibody molecules would be

$$A \text{ combined} = NB - \frac{\overline{NB}^2}{4A}.$$

If $R = \dfrac{\text{mol. wt. antibody}}{\text{valence antibody}} \times \dfrac{\text{mol. wt. antigen}}{\text{valence antigen}}$,

this formula reduces to

$$\text{Mg. antibody pptd.} = 2Rx - \left(\frac{R^2x^2}{A}\right),$$

where x = mg. antigen added.

If the valence of antibody is M, then

$\left(\dfrac{MA - NB}{MA}\right)^M A$ = number of uncombined antibody molecules.

$A - \left(\dfrac{MA - NB}{MA}\right)^M A$ = number of combined antibody molecules.

The number of antibody molecules having 0, 1, 2, and 3 . . . M groups combined is given by the successive terms of the binomial expansion:

$$\left(\frac{MA - NB}{MA} + \frac{NB}{MA}\right)^M.$$

The second case to be considered is one in which the reaction is reversible and an equilibrium is set up between free and combined reactive groups. The system must be treated as a heterogeneous system in which groups upon molecules in solution are in equilibrium with free and combined reactive groups upon surfaces. As before all of the reactive groups upon the antigen are considered to be alike.

If the valence of antibody is 1, equilibrium would be established between the number of free and combined groups upon the antigen, which is considered to form the surface phase, and the concentration of antibody in solution.

If A = Total number of antibody molecules in the system.
B = Number of antigen molecules added.

N = Valence of antigen.
x = Number of antigen groups in combination, and also
= Number of antibody groups in combination.
$(NB - x)$ = Number of free antigen groups.
$(A - x)$ = Number of free antibody molecules.

at equilibrium,

$$(NB - x)\frac{(A - x)}{V} = kx.$$

This equation reduces to a form which is the same as that derived by Ghosh[3] with the exception that it includes a volume term.

If the valence of antibody is 2, the groups upon either the antigen or antibody may be considered to constitute the surface phase which is in equilibrium with the reactive groups upon the molecules in solution. The reaction may be treated the same as in the case of the irreversible reaction with the exception that the number of antigen groups in combination will not be NB, the total number of antigen groups added, but will be some smaller number x determined by the magnitude of the dissociation constant. If there is a random distribution of x combined groups between the antigen and antibody molecules in the system,

$\left(\frac{2A - x}{2A}\right)^2 A$ = Number of uncombined antibody molecules.

$2\left(\frac{2A - x}{2A}\right)\left(\frac{x}{2A}\right)A$ = Number of antibody molecules with one group combined

$\left(\frac{x}{2A}\right)^2 A$ = Number of antibody molecules with both groups combined.

$(NB - x)$ = Number of free antigen groups.

If equilibrium is established in the system as a whole it will also be established in each step of the reaction. The first step will be the reaction of *one group* of an antibody molecule in solution with a reactive group upon the antigen.

$$A + b \rightleftharpoons Ab$$

The rate of combination will be proportional to the *concentration* of antibody groups in solution times the *number* of uncombined antigen groups. The rate of dissociation will be proportional to the number of antibody molecules with one group combined. At equilibrium

$$(NB - x)\left(\frac{2A - x}{2A}\right)^2\left(\frac{2A}{V}\right) = k\left(\frac{2A - x}{2A}\right)\left(\frac{x}{2A}\right)(2A) .$$

This simplifies to the expression,

$$(NB - x)\,(2A - x) = kVx.$$

If the antibody groups are considered to constitute the surface phase in equilibrium with the antigen groups on the antigen molecules in solution, the equation involved becomes

$$(2A - x)\left(\frac{NB - x}{NB}\right)^{N}\left(\frac{NB}{V}\right) = k\left(\frac{NB - x}{NB}\right)^{N-1}\left(\frac{x}{NB}\right)NB$$

which also simplifies to the same form.

The amount of antibody precipitated will be $x - \frac{x^2}{4A}$ instead of $NB - \frac{\overline{NB}^2}{4A}$, x being defined by the equation given above. If the dissociation constant is small the difference between these two values becomes experimentally significant only in the equivalence zone where the excess of antibody is small. Except in this region the reaction may be satisfactorily expressed by the formula,

$$\text{Mg. antibody pptd.} = 2Rx - \frac{k^2x^2}{A}$$

where x is the mgs. of antigen added.

In these cases all of the reactive groups upon the antigen were assumed to be alike and the reactivity of one group was not affected by the reaction of the other groups on the same molecule. The necessity of these assumptions can be tested.

Let it be assumed that antigen has four groups that react with the same *bi-valent* antibody to form compounds that have different dissociation constants.

If A = Number of antibody molecules present.

B = Number of antigen molecules present.

x, y, z, and u are the numbers of the different groups that have reacted.

At equilibrium,

$$\begin{aligned}(2A - x - y - z - u)(B - x) &= k_1Vx\\ (2A - x - y - z - u)(B - y) &= k_2Vy\\ (2A - x - y - z - u)(B - z) &= k_3Vz\\ (2A - x - y - z - u)(B - u) &= k_4Vu\end{aligned}$$

If values are assigned to the k's, relative values can be computed for the other terms in these equations. The amount of antibody combined will be,

$$(x + y + z + u) - \frac{(x + y + z + y)^2}{4A}$$

As shown in TABLE 1, the relative amounts of antibody combined by antigen which has 4 groups reacting with antibody with dissociation constants 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2}, are compared with the amount

precipitated by antigen in which all valences are alike and have a dissociation constant of 10^{-5}.

TABLE 1

Antigen added	Antibody combined	
	Mixed antigen	Simple antigen
0.1	0.1900	0.1900
0.2	0.3600	0.3600
0.3	0.5100	0.5100
0.616	0.8518	0.8526
0.764	0.9434	0.9443
0.802	0.9600	0.9608

From the data summarized in TABLE 1, it can be seen that it would be impossible to distinguish experimentally between the two systems.

Another case has been considered in which it is assumed that all of the groups upon the antigen are alike, but that the reaction of each group lowers the tendency for the remaining groups to react with antibody. The reaction is considered to take place in steps and the dissociation constant is assumed to increase by a factor of 10 for each step. If x, y, z, and u represent the number of molecules of antigen with 1, 2, 3 and 4 reactive groups combined, the equilibrium equations would be:

$$(2A - x - 2y - 3z - 4u) \cdot 4(B - x - y - z - u) = k_1 V x$$
$$(2A - x - 2y - 3z - 4u) \cdot 3(x) = 2k_2 V y$$
$$(2A - x - 2y - 3z - 4u) \cdot 2(y) = 3k_3 V z$$
$$(2A - x - 2y - 3z - 4u) \cdot (z) = 4k_4 V u$$

Relative values for A, B, x, y, z and u can be computed from these equations and the amount of bivalent antibody bound by different amounts of antigen can be calculated.

In TABLE 2 is depicted a comparison between the relative amounts of

TABLE 2

Antigen added	Antibody combined	
	Variable k $k = 10^{-5} - 10^{-2}$	Fixed k $k = 10^{-5}$
0.231	0.4090	0.4092
0.335	0.5566	0.5572
0.405	0.6456	0.6458
0.536	0.7518	0.7536
0.670	0.8910	0.8934
0.850	0.9738	0.9770

antibody combined with antigen in this system and the amount bound by antigen with a fixed dissociation constant.

From the results set forth in TABLE 2, it would be impossible to distinguish analytically between the two systems in the region of antibody excess.

It has been shown that it makes little difference quantitatively whether or not all of the groups upon the antigen react alike. What would be the effect if the two reactive groups upon the antibody molecule were different in their reactivity? Assume that the antibody molecule has two reactive groups A_1 and A_2 that react with the same groups upon the antigen molecules, and that the dissociation constants k_1 and k_2 are of different magnitudes.

If A = Number of antibody molecules.
B = Number of antigen molecules.
x = Number of A_1 groups combined.
y = Number of A_2 groups combined.
at equilibrium,

$$(A - x)\ (NB - x - y) = k_1 V x.$$
$$(A - y)\ (NB - x - y) = k_2 V y.$$

$$\text{Mols. antibody combined} = x + y - \frac{xy}{A}.$$

Assuming values for A, k_1, and k_2 the relative values for NB, x and y can be calculated. FIGURE 1 compares the curve obtained for a system where $A = 1.0$, $k_1 = 10^{-5}$, $k_2 = 10^{-4}$ with the curve obtained if both of the antibody groups were alike. One system could be distinguished from the other experimentally.

If the antibody has two reactive groups reacting with different kinds of groups upon the antigen molecule,

N_1 = Number of the first kind of group on the antigen molecule.
N_2 = Number of the second kind of group on the antigen molecule.
x = Number of A_1 groups combined.
y = Number of A_2 groups combined.

$$(A - x)\ (N_1 B - x) = k_1 V x.$$
$$(A - y)\ (N_2 B - y) = k_2 V y.$$

If k_1 and k_2 are small, x approximates $N_1 B$ and y approximates $N_2 B$, except near the equivalence point.

$$\text{Mols. antibody combined} = (N_1 + N_2)B - \frac{N_1 N_2 B^2}{A}.$$

If $N_1 = N_2$, the equation becomes identical with the one obtained for antibody with both groups alike. For all other values for N_1 and N_2 the amount of antibody combined will be greater than for the simple system.

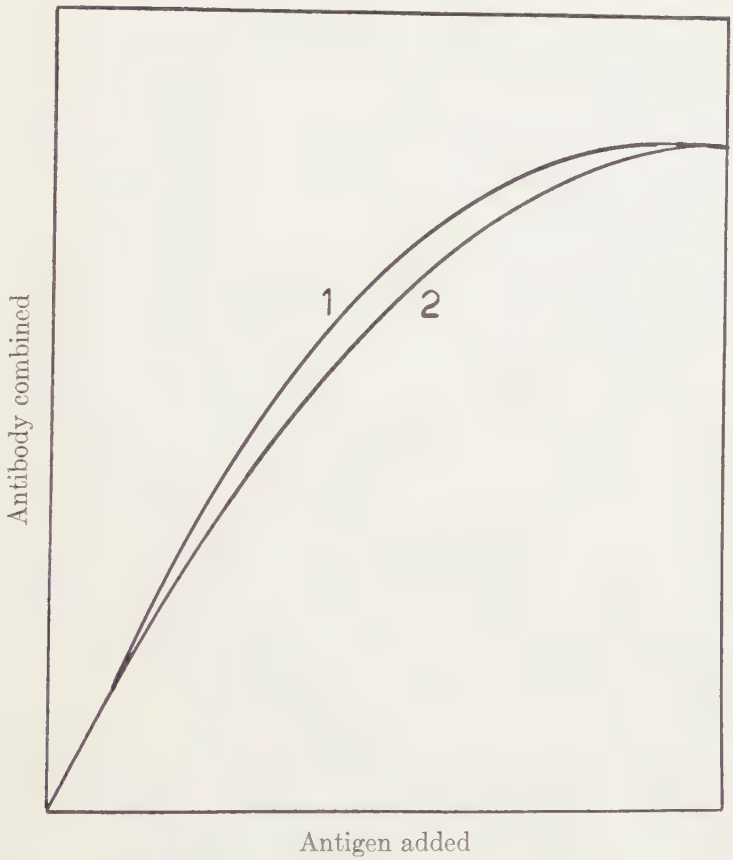

FIGURE 1. Reaction of non-homogeneous antibody. The curves show the effect of a difference in the reactivity of the two reactive groups of bi-valent antibody.

1. $k_1 = 0.1k_2$;
2. $k_1 = k_2$.

FIGURE 2 shows the types of curves obtained when $N_1 = N_2$ and $N_1 = 5N_2$.

The antiserum formed by immunizing a rabbit with an antigen may contain a mixture of antibodies reacting with different specific groups upon the antigen molecule. A great number of variations are possible in such a system.

1. If the number and position of the reactive groups upon the antigen permit the independent reaction of each group the amount of antibody precipitated from a mixed antiserum will be the sum of the amounts calculated for each individual system. If A_1, A_2, *etc.*, represent the amount of the different antibodies present and N_1, N_2, *etc.*, represent the

number of the different reactive groups upon an antigen molecule, the amount of monovalent antibody combined will be

$$N_1B + N_2B + etc., \text{ (if } k_1, k_2, etc., \text{ are small)}$$

as long as an excess of all kinds of antibody is present. If the different antibodies are present in varying amounts so that some of them are completely precipitated while others are present in excess this expression becomes

$$\text{Antibody combined} = A_1 + A_2 + N_3B + \ldots . etc.$$

If antibody combined is plotted against antigen added a graph made up of several straight line facets would result.

If the antibody is bi-valent the amount combined will be

$$N_1B - \frac{\overline{N_1B}^2}{4A_1} + N_2B - \frac{\overline{N_2B}^2}{4A_2} + etc,$$

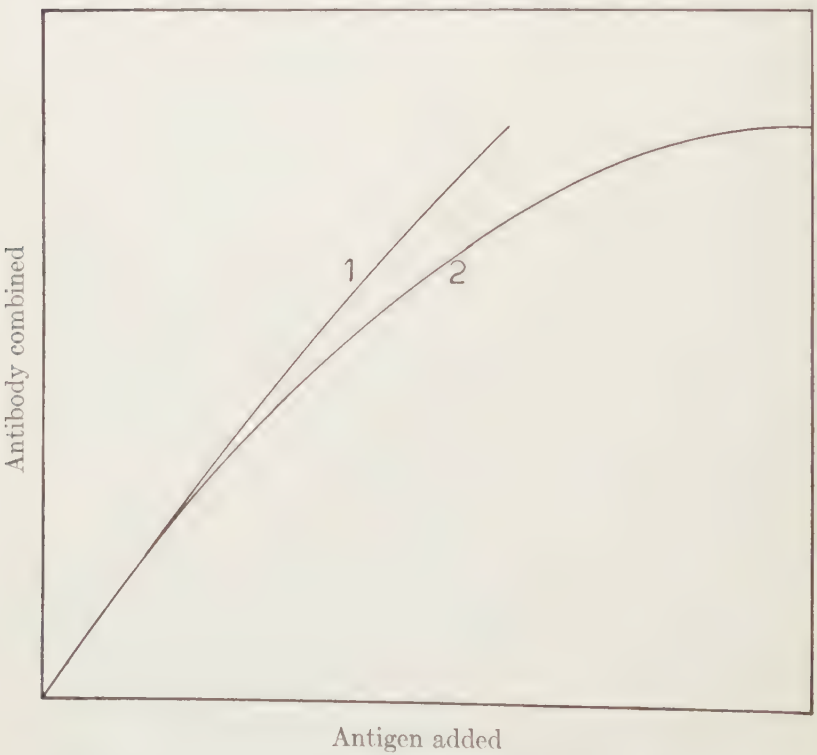

FIGURE 2. Reaction of non-homogeneous antibody. The antibody molecule is assumed to have two groups that react independently with two different kinds of groups upon the antigen. The curves show the effect of a difference in the relative numbers of the antigen groups.

1. $N_1 = 5N_2$;
2. $N_1 = N_2$.

as long as an excess of all kinds of antibody is present. If one of them is exhausted while an excess of the others is present the expression becomes,

$$A_1 + N_2B - \frac{\overline{N_2B}^2}{4A_2} + etc.$$

2. If the number or position of the reactive groups upon the antigen do not permit the independent reaction of each group many different cases are possible. The only case to be considered in detail is one in which there are two kinds of groups upon the antigen and the number and position of the groups prevent independent reaction of the two kinds of antibody. There will be a competition between the two kinds of antibody for the available groups. If $k_1 = k_2$ the system will react like a system in which all of the antibody is alike. If $k_1 \neq k_2$ the following relationships should hold for bi-valent antibody.

Let $N_1 = N_2 = N$ the number of available groups on the antigen.
$A_1 =$ Mols. of antibody 1 present.
$A_2 =$ Mols. of antibody 2 present.
$x =$ Number of groups of antibody 1 in combination.
$y =$ Number of groups of antibody 2 in combination.

At equilibrium,

$$(2A_1 - x)\ (NB - x - y) = k_1Vx.$$
$$(2A_2 - y)\ (NB - x - y) = k_2Vy.$$
$$y = \frac{2k'A_2x}{2A_1 - (1 - k')x} \text{ where } k' = \frac{k_1}{k_2}$$
$$\text{Antibody combined} = x - \frac{x_2}{4A_1} + y - \frac{y^2}{4A_2}$$

The values obtained for systems in which

$A_1 = A_2$, $k_1 = 10^{-6}$ and $k_2 = 10^{-4}$ and
$3A_1 = A_2$, $k_1 = 10^{-6}$ and $k_2 = 10^{-4}$ are shown in FIGURE 3.

The theory presented here does not predict the way in which all antigen-antibody systems will react. No single equation could be adequate for all possible variations in such a system. Its value lies in the fact that it permits logical deductions to be made concerning the chemical nature of the antigen and antibody molecules from a quantitative study of their interaction.

The data obtained by Heidelberger and Kendall[1c] for the reaction of crystalline egg albumin with the antisera obtained after different periods of immunization of the same rabbit are analysed in the following discussion.

The theory predicts that the maximum ratio of antibody and antigen combined should be

$$R_{max} = \frac{\text{Molecular weight of antibody}}{\text{Molecular weight of antigen}} \times \text{valence of antigen,}$$

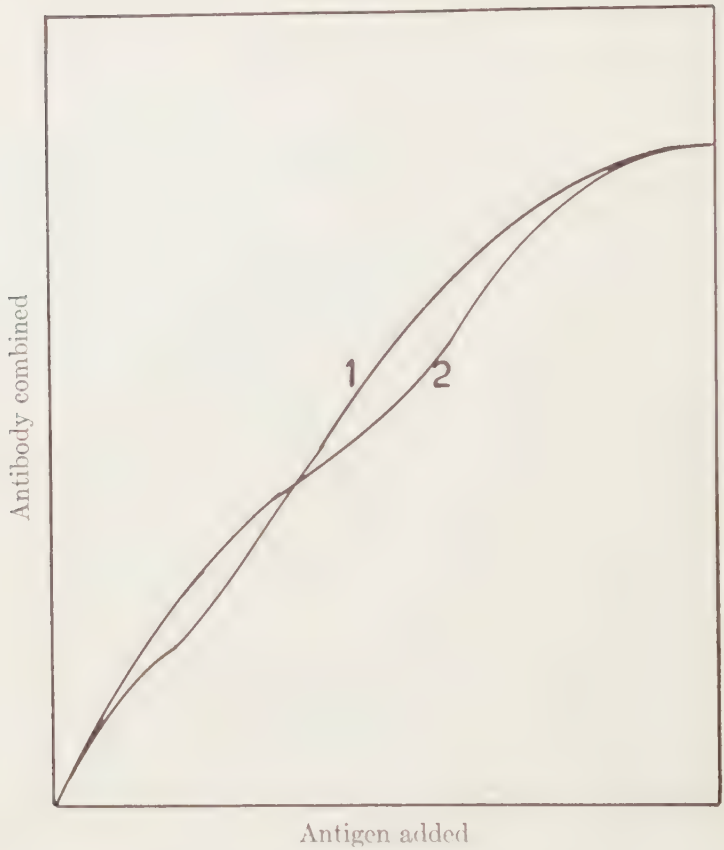

FIGURE 3. Reaction of non-homogeneous antibody. The antiserum is considered to contain two species of antibody molecules reacting with the same antigen groups.

1. $3A_1 = A_2$, $k_1 = 10^{-6}$, $k_2 = 10^{-4}$;
2. $A_1 = A_2$, $k_1 = 10^{-6}$, $k_2 = 10^{-4}$.

Since the valence of antigen is an integral number this relationship predicts that the maximum ratio must be one of a limited number of values. Assuming molecular weights of 160,000 and 40,000 for the antibody and E. A., the possible ratios would be some multiple of 4. The change in ratio as the amount of added antigen is increased can be due either to dissociation, which would leave increasing numbers of antigen groups uncombined with antibody as the concentration of free antibody in the supernatent diminished, or to polyvalency of the antibody molecules. If the valence of antibody is one the change in ratio must be due to dissociation and the reaction should follow the equation,

$$(A - x)(rB - x) = kVx,$$

where A = mgs. total antibody in the system.
B = mgs. antigen added.
r = maximum ratio.
x = mgs. antibody combined.

Since a high dissociation constant would make experimental determination of the value of A impossible this equation contains 3 constants, A, r and k, which must be evaluated from the experimental data. This has been done for this system. Table 3 shows that values can be found

Table 3

Anti-Egg Albumin Rabbit Serum Number 387

$(A-x)\ (rB-x)=kvx$

1st Course $A=1.00$ $r=24$ $k=.23$ $v=2$ ml.			2nd. Course $A=1.3$ $r=24$ $k=.135$ $v=2$ ml.			3rd. Course $A=1.6$ $r=24$ $k=.08$ $v=2$ ml.		
Ea.	Antibody precipitated		Ea.	Antibody precipitated		Ea.	Antibody precipitated	
mg. N	Found *mg.* N	Calculated *mg.* N	*mg.* N	Found *mg.* N	Calculated *mg.* N	*mg.* N	Found *mg.* N	Calculated *mg.* N
.009	0.15	0.14	.015	0.29	0.32	.03	0.64	0.63
.015	.22	.23	.05	0.79	0.79	.049	0.96	0.94
.025	.35	.35	.088	1.06	1.04	.079	1.24	1.27
.040	.49	.50	.098	1.08	1.10	.082	1.29	1.30
.050	.58	.58	.118	1.10	1.12	.088	1.33	1.33
.065	.68	.67	.127	1.15	1.14	.098	1.37	1.37
.074	.72	.70	Volume 9 ml.			Volume 8 ml.		
.082	.75	.73	.098	1.05	0.76	.079	1.28	0.95

for these constants that give good agreement between the experimental data and the calculated values. The values of the constants for the different antisera are reasonable. As might be expected the value of A increases as the period of immunization of the rabbit becomes longer. The value of k decreases, *i.e.*, a long period of immunization increases the avidity with which antibody combines with antigen. The value for r remains constant throughout and indicates that the number of reactive groups upon the antigen molecule is 6. It would appear therefore that the reaction between egg albumin and its homologous antibody could be explained upon the assumption of monovalent antibody. However this assumption requires values for the dissociation constant k that would

lead to pronounced changes in the amount of antibody combined should the volume be changed. The data given in TABLE 3 shows that a change in volume does not have the predicted effect. Therefore, in spite of the agreement between experimental and calculated values, the course of the reaction in this system cannot be explained by the assumption that the valence of antibody is one.

It was shown[1c] that, although the antibody obtained after the first course of immunization reacted with egg albumin as if it were homogeneous and had a valence of 2, the antibody obtained from later bleedings did not. Evidence was presented which indicated that the antibody in these sera was not homogeneous, and "that in the later stages of immunization antibody is formed which is reactive with a larger number of chemically distinct groupings on the Ea molecule than was the antibody produced in the earlier stages of immunization." The theoretical treatment of the problem given in this paper permits the testing of this assumption.

An egg albumin molecule was assumed to have two kinds of reactive groups reacting independently with different bi-valent antibody molecules. The number of the first kind of group, which stimulated the rapid formation of antibody in the rabbit, was assumed to be 4. The number of the second kind which stimulated slow antibody response, was assumed to be 2. The relative proportions of the two antibodies was considered to be 1.00 to 0.00, after the first course of immunization, .90 to .10 after the second, and .75 to .25 after the third. FIGURE 4 shows that the experimental results are adequately explained by these assumptions.

Although these assumptions are not the only ones that will lead to the numerical agreement between the experimental and theoretical values, they represent the simplest system that will give agreement. The probability of their correctness is increased by the observation ([1c]: TABLE 5) that, if part of the antibody in an antiserum obtained by long immunization is removed by absorption, the remainder of the antibody reacts as if the antigen had a valence of 4.

In the development of the theory up to this point no assumptions have been made as to the cause of the insolubility of the compound between antigen and antibody. The assumption was simply that the compound was insoluble. The relationship between combined antigen and antibody should hold regardless of the factors responsible for the insolubility of the compound.

Two general theories have been proposed to account for the precipitation of the antigen-antibody complex. One theory postulates that the combination of antigen and antibody leads to the formation of particles

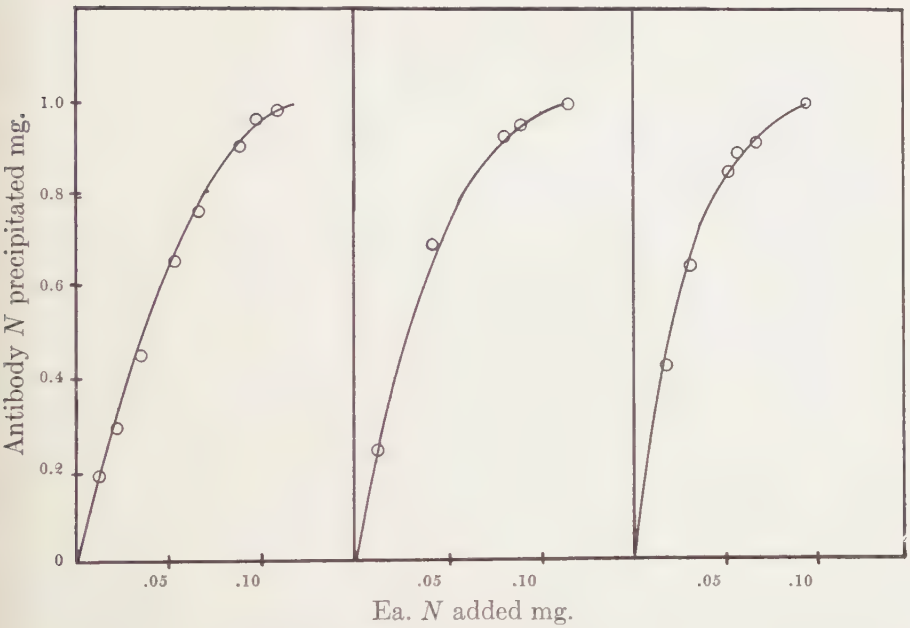

FIGURE 4. Reaction of complex antigen with antibody mixtures. The antigen is assumed to have 4 groups of type A and 2 of type B. The curves are calculated for different mixtures of antibody. 1. 100% A, 2. 90% A, 10% B. 3. 75% A, 25% B. The circles represent experimental values obtained after different periods of immunization of a rabbit with egg albumin.

whose surfaces are more "hydrophobic" than the surfaces of the uncombined antigen and antibody molecules.[4] If sufficient salt is present to reduce the surface charge of these particles below a critical value, non-specific flocculation and precipitation takes place. The second theory postulates "*specific flocculation*" as well as specific combination.[5] The union of multivalent antigen and antibody should lead to the formation of large aggregates in which alternate molecules of antigen and antibody are linked together to form a lattice-like structure.

It must be recognized that the two theories are interrelated. The same surface forces which prevent non-specific flocculation would also act to prevent the chemical reaction of antigen and antibody groups. The surfaces bearing these groups must come into contact with each other before the reaction can take place. As the "coulomb forces" increase with the size of the aggregates these forces must be reduced below a critical value before specific combination can lead to the formation of large aggregates.

It should be possible to demonstrate mathematically whether or not the "specific aggregation" theory alone is sufficient to explain precipitation.

Let us consider a hypothetical system in which the antigen has N reactive groups all of which are alike and in which the antibody has a valence of 2. In the presence of an excess of antibody all of the antigen valences may be considered to be in combination. Part of the antibody molecules in combination will have only one group combined. It is evident that antigen molecules that are combined only with a singly-bound antibody would form large isolated molecules that would not be part of a large aggregate and so should not be precipitated. Molecules of antigen combined with a doubly bound antibody molecule may be part of a large aggregate even though most of the antibody molecules upon the antigen are bound with a single valence.

According to the theory presented here the number of antibody molecules which have a single group combined is given by the expression,

$$\frac{(2A - x)x}{2A},$$

where A is the number of antibody molecules present and x is the number of antigen groups in combination and is equal to NB, the number of antigen molecules if k is small. Since each of these molecules is bound to an antigen group this expression also gives the number of antigen groups bound to a singly bound antibody. This expression divided by x gives the fraction of the antigen groups so combined, and also the *probability* that any certain antigen group is so combined. $\left(\frac{2A - NB}{2A}\right)^N$ equals the probability that all of the groups upon a given antigen molecule of valence N are in combination with a singly bound antibody. $\left(\frac{2A - NB}{2A}\right)^N \cdot NB$ is the probable number of antigen groups not combined in a large aggregate and, as each of these groups is combined with an antibody molecule, it is also the number of antibody molecules so combined. The number of antigen molecules in the aggregates would be

$$\left[1 - \left(\frac{2A - NB}{2A}\right)^N\right]B.$$

As the number of antibody molecules combined has been shown to be $NB - \frac{NB^2}{4A}$, this number minus $\left(\frac{2A - NB}{2A}\right)^N \cdot NB$, represents the number of antibody molecules in the aggregates.

In FIGURE 5 curves are plotted which show the relative amount of antibody precipitated by various amounts of antigen. The first curve shows the amount of antibody that would be in combination and the other curves the amount that would be precipitated according to the specific aggregation theory, assuming antigen valences of 6, 4 and 2. It

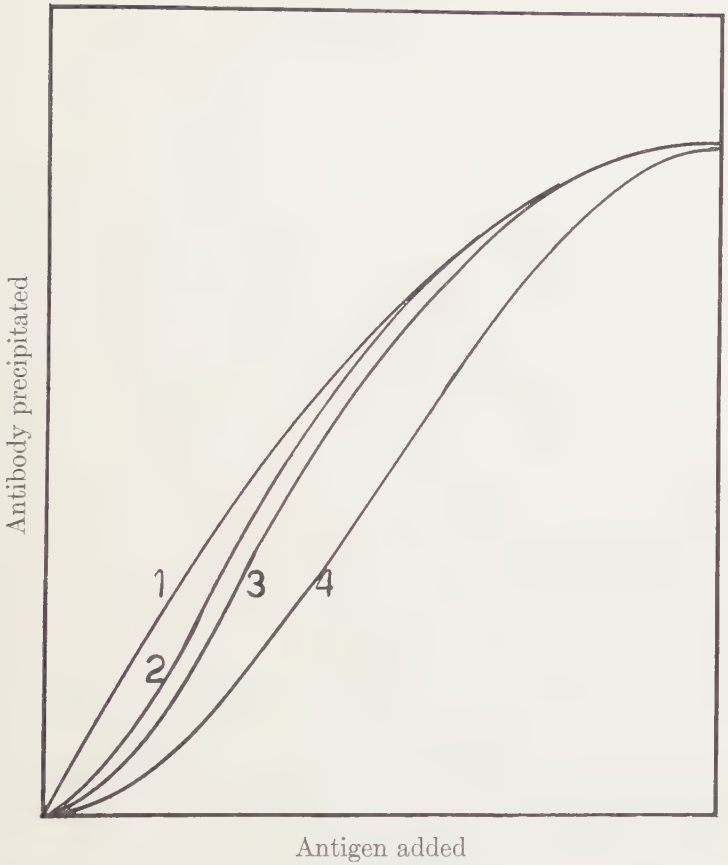

FIGURE 5. Antibody precipitated according to theory of "specific aggregation." 1. Antibody combined; 2. Antibody precipitated by antigen valence 6; 3. Antibody precipitated by antigen valence 4; 4. Antibody precipitated by antigen valence 2.

has previously been shown that the experimental values obtained for antibody precipitated in anti-protein rabbit systems agree with the values calculated upon the assumption of complete precipitation. The differences between these experimental values and those calculated for the theory of specific flocculation is greater than the uncertainty involved in the determination of the antibody precipitated. It would thus appear that specific flocculation alone is not sufficient to explain the precipitation of the antigen antibody complex. However, the calculated values were obtained upon the assumption that the antibody was homogeneous and that all of the reactive groups upon the antigen were alike. These conditions are probably never encountered in natural systems. It can

be shown that as little as ten per cent of a second antibody reacting independently with the antigen would lead to practically complete precipitation throughout the reaction. Final judgment concerning the adequacy of the theory of specific flocculation cannot be given until simpler systems have been studied in detail.

In contrast to its failure to give satisfactory agreement with the experimental data in the systems in which the antibody was formed in rabbits, the specific agglutination theory, modified in one particular, adequately explains the data obtained in toxin-antitoxin and certain protein-antiprotein systems where the antibody is formed in the horse.

Pappenheimer, Lundgren, and Williams[6] pointed out that "the exceptional character of the antitoxin flocculation reaction lies chiefly in the soluble inhibition zone in the region of antitoxin excess." They suggested that this peculiarity may be due to an "unsymmetrical distribution" of the antitoxic groups upon the surface of the antitoxin molecule. Any assymmetry in the position of these reactive groups which would have any effect upon the reactivity of the groups would be reflected in a difference in the dissociation constants for the reaction of the two groups. Therefore, let it be assumed that an antitoxin molecule has two different reactive groups, A_1 and A_2, which react either with the same or with different reactive groups upon the toxin molecule. If the number of each reactive group on the toxin is equal to or is greater than N, (the maximum number of antitoxin molecules that can be arranged on the surface of the toxin molecule) it makes no difference which assumption is made. In one case the different antitoxic groups would be competing for the same toxin groups and in the other for the same spaces on the toxin molecule. At equilibrium:

$$(A - X)\,(NT - X - Y) = k_1VX.$$

$$(A - Y)\,(NT - X - Y) = k_2VY.$$

where

A = Number of antitoxin molecules in the system.

T = Number of toxin molecules in the system.

X = Number of A_1 antitoxic groups that have reacted.

Y = Number of A_2 antitoxic groups that have reacted.

N = Valence of toxin. V = Volume.

k_1 = Dissociation constant for A_1 groups.

k_2 = Dissociation constant for A_2 groups.

$(A - X)$ Number of unreacted A_1 groups in the system.

$(A - Y)$ Number of unreacted A_2 groups in the system.

$(NT - X - Y)$ Number of unreacted toxin groups in the system.

From these equations the relationships,

$$Y = \frac{k'Ax}{A + (k' - 1)x} \text{ where } k' = \frac{k_1}{k_2}, \text{ and}$$

$$NT = x + y + \frac{k_1 V x}{A - x}$$

may be obtained.

If X and Y are expressed as fractions of the total antitoxin present, the product XY gives the fraction of the antitoxin with both reactive groups in combination, and XYA the number of antitoxin molecules so combined.

Only those toxin molecules which are combined with at least one antitoxin molecule that has both groups in combination, can be part of the aggregate and thus be in the precipitate.

The number of molecules of toxin of valence N bound by XYA molecules of antitoxin is given by the formula:

$$T' = \left[1 - \left(1 - \frac{2xyA}{NT}\right)^N\right]T.$$

This formula may be obtained either by an application of the law of mass action to this reaction, or by an application of the law of probability if it is assumed that a perfectly random distribution of the antitoxin molecules exists on the surface of the toxin.

If the proportion of the toxin groups in combination with antitoxin is the same in the precipitate as in the system as a whole the number of antitoxin groups in the precipitate would be

$$\left(\frac{x + y}{NT}\right)T'.$$

The number of antitoxin molecules in the precipitate would be

$$N\left(\frac{x + y}{NT}\right)T' - XYA.$$

These relationships enable one to calculate the number of toxin and antitoxin molecules in the floccules for any value of k_1, k_2 and N. These numbers may be converted into experimental units by assuming that the total antitoxin nitrogen in the system is equivalent to one molecule of antitoxin. The toxin nitrogen equivalent to one valence unit of toxin would then be

$$\frac{\text{Molecular wt. Toxin x \%N} \cdot \text{Valence Antitoxin}}{\text{Molecular wt. Antitoxin x \%N} \cdot \text{Valence Toxin}} \times \text{Total Antitoxin } N\cdot$$

and the nitrogen equivalent to one molecule of toxin, N times this value.

Pappenheimer and Robinson[7] give values for a system containing 0.48 mg. of antitoxin nitrogen. Using their values for the molecular

weight and nitrogen content of toxin and antitoxin the theoretical curve for toxin of valence 4 has been calculated when values of 10^{-5} and 10^{-4} are assumed for the two dissociation constants.

FIGURE 6 compares the theoretical curve with the experimental values and shows that good agreement is obtained up to the point where the precipitate begins to dissolve in an excess of toxin. No attempt has been made to explain the reaction beyond this point.

It would appear that the differences in the reaction of an antigen with antibody formed in a rabbit and the reaction of diphtheria toxin with

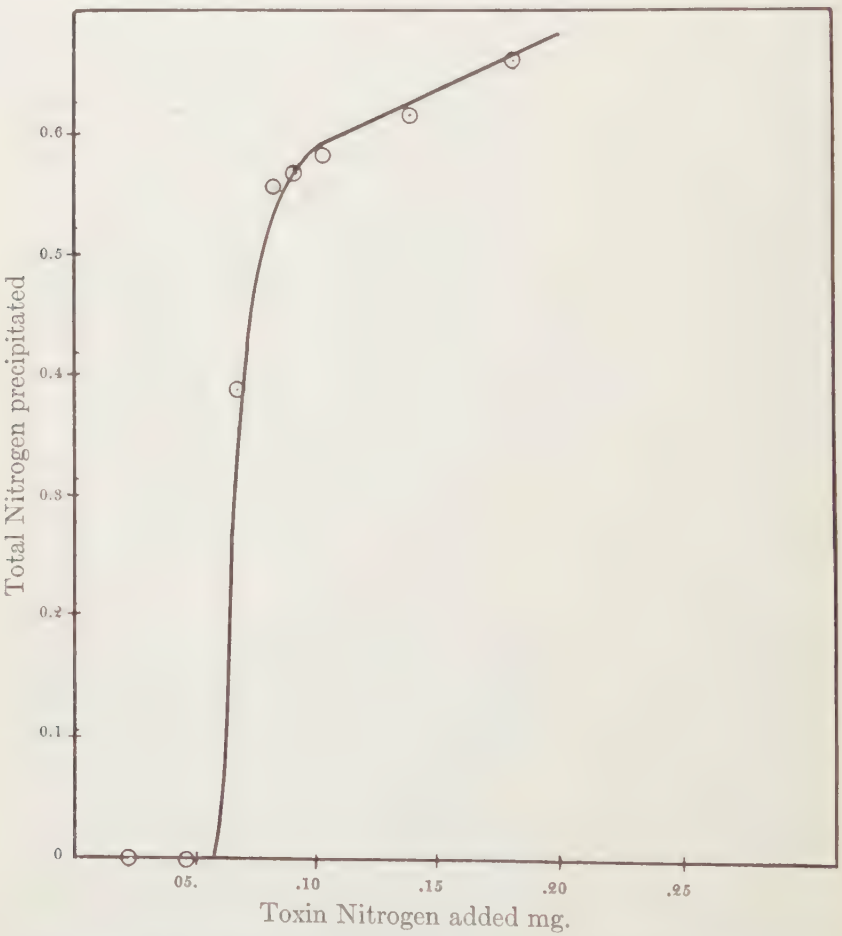

FIGURE 6. Diphtheria toxin-antitoxin flocculation reaction. Curve calculated from theory. Circles represent experimental points.

antitoxin formed in a horse can be adequately explained by assuming that rabbit antibody has two reactive groups which are alike and horse antitoxin has two groups which are different in their reactivity.

SUMMARY AND CONCLUSIONS

It has been shown that the equation,

$$y = 2Rx - \frac{R^2x^2}{A},$$

derived by Heidelberger and Kendall[1a] by an application of the mass law to the precipitin reaction can also be derived by assuming that antibody has a valence of 2 and that there is a random distribution of the antibody groups between the available reactive groups upon the antigen. Although the equation is strictly true only in an irreversible system where all of the groups upon both the antigen and antibody molecules react alike, the effect of dissociation in a reversible system has been shown to be negligible except in the region of the equivalence point if the dissociation constant is small. Great variation can exist in the reactivity of individual antigen groups without producing any great deviations from this formula as long as the antibody is homogeneous. However, if the antibody is not homogeneous significant differences from this formula would be predicted.

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EQUINE ANTIHEMOCYANIN

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INTRODUCTION

Analysis of antigen-antibody compounds, especially the soluble ones, is facilitated and made more precise and dependable when the antigen (or antibody) contains a structure such as a metallic ion,[1–3] dye,[4] or isotope[5] that is accurately measurable in low concentration. The copper-content, the large molecular size, and the high antigenicity of hemocyanin are properties offering considerable advantages for such analyses.[6] It is desirable to study the characteristics of equine antibody because a large variety of therapeutic sera are derived from horses and bleedings of a size ample for adequate study can be obtained during the course of immunizing individual animals. In 1935 we obtained only a trace of precipitin from a horse immunized with *Limulus* hemocyanin by the Massachusetts Antitoxin and Vaccine Laboratory. Pappenheimer[7] succeeded in producing antiovalbumin in a horse and we have recently obtained a powerful equine antihemocyanin some of whose properties are herein described.

Preparation of the Antigen

Hemocyanin from the blood of *Busycon canaliculatum* was purified by three successive precipitations near the isoelectric point (pH 4.5). The material was dissolved with the minimal amount of alkali, adjusted to about 1 per cent concentration, rendered isotonic by addition of salt, and passed through a Berkefeld filter.

None of the numerous lots of hemocyanin was found to be sterile after Berkefeld filtration, unless a preservative (Merthiolate) had been added. They all contained a coliform bacillus which was cultivable but which in broth would not pass the filter. The hemocyanin thus prepared had the usual nitrogen/copper ratio of about 60, varying somewhat in different lots. From this, and from direct observation of smears, it was apparent that only a very small fraction of the nitrogen in our preparations could have been due to bacteria. Also, before filtration the preparations were centrifuged, which would reduce the number of organisms. This contamination of our antigen must be mentioned although it appears to be unimportant.

Preparation of Antiserum

The Lederle Laboratories very kindly immunized a horse (A633) with the hemocyanin. All injections except the first were given subcutaneously, starting with very small quantities. The amount of the third bleeding was small. Following the fourth bleeding the horse was in such poor condition that he was exsanguinated. Dates and doses are given in TABLE 1.

TABLE 1

HORSE A633

INJECTED WITH HEMOCYANIN OF *Busycon canaliculatum**

Date	Dose	Date	Dose†
1939	*ml.*	*1939*	*ml.*
6/29	1	8/25	20
7/5	1	8/30	Bleeding 1
7/10	2	8/31	20
7/14	3	9/5	20
7/19	4	9/12	Bleeding 2
7/24	5	9/26	40
8/1	5	9/29	15
8/4	5	10/3	15
8/8	10	10/6	15
8/11	10	10/13	Bleeding 3
8/17	20	10/16	Bleeding 4
8/22	20	10/20	Bleeding 5

* The last 3 injections contained 4 per cent protein; all others 1 per cent. Total hcy-N injected was 566 mg.

† Assuming that the horse contained 25 L of "serum" then at the time of the first bleeding 34 grams of circulating antibody had been produced per gram of hemocyanin injected, the corresponding figures for the 2nd and 4th bleedings are 82 and 57 (assumed N-factors: ab, 6.25, hcy, 6.45).

Necropsy revealed a "badly abused liver, about to rupture." Our previous attempt to immunize a horse against *Limulus* hemocyanin also led us to suspect that hemocyanin is toxic to horses. The animal died "with hallucinations." Rabbits, on the other hand, almost invariably tolerate the injections well. Purified *Busycon* hemocyanin does not agglutinate the erythrocytes of horse or rabbit.

The successive bleedings had antibody-nitrogen contents of 0.19, 0.74, 1.15, and 0.94 mg. ml. when redetermined after 15 months with the pooled lot of hemocyanin now being used. Figures obtained when the sera were fresher were but slightly larger; the antibody appears to be pretty stable. A sample of serum from the fourth bleeding, lyophilized immediately, now contains 1.32 mg. *ab*-N/ml. The antibody was

almost entirely in the "pseudoglobulin I" fraction of the serum-proteins, as thrown down with sodium sulphate according to Howe.

The coliform organism grown from the hemocyanin was agglutinated by the immune sera in dilutions up to 1:25 or 1:50 but there was a *decrease* in the agglutinative potency of the sera from the later bleedings, and sera from two other horses not injected with hemocyanin also caused about the same degree of agglutination. Three samples of rabbit-anti-hemocyanin contained no agglutinin for the organism. Thus, this contaminant appeared to have no antigenic significance for the system under investigation.

SEROLOGICAL CHARACTERISTICS

The most prominent feature of the behavior of this antibody is its similarity to diphtheric antitoxin and equine anti-ovalbumin, in that no visible reaction occurred in the region of excessive antibody, in contrast to antihemocyanin or antitoxin from the rabbit. However, the zone in which visible reactions did occur was very much broader than was the case with either of the other equine antiproteins mentioned. This will be apparent from the determinations of nitrogen in precipitates made with various mixtures of antigen and antiserum, shown in TABLE 5 and FIGURES 1, 2 and 3.

Precipitative Reactions

In the zone of equivalence or of slight excess of antigen, no significant difference was observed in the amounts of precipitate obtained after 2 hours at 37°, or at 24 hours or 48 hours in the icebox (TABLE 2).

TABLE 2

SHOWING COMPLETENESS OF PRECIPITATION AFTER TWO HOURS IN ZONE OF EQUIVALENCE AND OF SLIGHT ANTIGEN-EXCESS. AVERAGE OF DUPLICATE ANALYSES.

Time	Temp.	Hcy-N added	Serum	Total N	Hcy-N in super.
hr.	*C.*	γ	*ml.*	γ	γ
2	37°	274	1.	610	—
24	6°	274	1.	582	—
48	6°	274	1.	615	—
2	37°	274	0.25	152	9
24	6°	274	0.25	185	6
48	6°	274	0.25	139	10

Several experiments were set up in which the same amounts of reagents in varying volumes (from 1 to 27 ml.) were used. The amount of pre-

cipitate formed from mixtures in proportions corresponding to the mid-point of the equivalence-zone diminished progressively with increasing volume. The decrease seemed to be greater than could be accounted for by simple solubility of the precipitate (TABLE 3).

TABLE 3

INFLUENCE OF CONCENTRATION UPON AMOUNT OF PRECIPITATE
$H633_5$ 0.5 ml. + 500 γ HEMOCYANIN-N (IN EQUIVALENCE-ZONE)

Volume				Total ppt-N	
ml.					
1.	All		Washed each	1190	Diff.
5.	at		sediment with	977	−213
25.	37°		1 ml. ss after	869	−321
1.	120′	Sed. + 5. ml.	refrigeration	1105	− 85
1.		Sed. + 25. ml.	over night	1041	−149

The solubility in saline of a precipitate made in the region of slight antibody-excess and already washed three times was determined by repeated 3-day extraction in the icebox. It was found that the solubility depended on the total amount of precipitate present, and on the number of previous extractions with saline. The solubility of a pure homogeneous substance should not depend on either of these factors. The solubility was observed to vary from about 40 γ N/ml. saline to 0.1 γ N/ml. The solubility after 6 extractions was decreasing much less rapidly than at first, but had not become stationary. Beyond this point determinations required volumes too large to be practicable (TABLE 4). We may regard the solubility-behavior as evidence of the non-uniformity of the precipitate.

TABLE 4

SOLUBILITY IN SALINE OF THRICE-WASHED PRECIPITATES
(γ N/ml)

Extraction	Volume of extractant	Ml. saline/g precipitate-N
	333 ml.	1660 ml.
1	37.9 γ	21.1 γ
2	16.9	33.6
3	8.2	2.4
4	5.6	2.6
5	3.6	1.1
6	0.4	0.1

Antihemocyanin when heated to 70° for 30 minutes lost its power of precipitating hemocyanin. Such heated serum also greatly retarded the rate of flocculation of hemocyanin with unheated serum, but did increase the amount of nitrogen in the precipitate from such a mixture. In one experiment the increase in amount of precipitate-N was considerably greater than the antibody-N present in the serum before heating. Heated heterologous antisera gave no increase detectable by simple inspection of the centrifuged precipitates. Quantitative data will form the basis of a subsequent report. It is of interest to note the essential similarity of the behavior of this heated serum and the non-precipitating antibody ("univalent"[7, 8]) present in Pappenheimer's horse during the early stage of immunization with ovalbumin. Has the effect of heating been to render the antibody "univalent" or to impair its firmness of union with antigen, perhaps by some reduction in the complexity of its combining group?

TABLE 5

PRECIPITATION OF HEMOCYANIN WITH LYOPHILIZED SERUM A633$_4$ CALCULATED TO 1 ML. OF ANTISERUM. (DUPLICATE ANALYSES).

Hcy-N added	Total N	Hcy-N detected in		Ab-N detected in		"R"[2] ab/ag in ppt.	Mol. R[3] ab/ag
		Ppt.	Super.	Ppt.	Super.		
γ	γ	γ	γ	γ	γ		
149	38	?	0		+		
196	770	All?	0	574	+	2.93	147
294	1322[1]	All?	0	1028	+	3.50	175
392	1560	All?	0	1168	+	2.98	149
490	1760	All?	0	1270	0	2.60	130
588	1912	All?	0	1324	0	2.26	113
686	1971	All?	0	1285	0	1.87	94
784	2108	All?	0	1324	0	1.69	85
980	2282	All?	Tr.	1302	0	1.33	67
1383	2584	1361	22	1223	0	.89	45
1844	3042	1790	54	1252	0	.70	35
2305	2854	1813	292	941	0	.49	25

[1] One determination lost.
[2] The ratios outside the zone of equivalence are not reliable; see text.
[3] Taking m. w's. as 160,000 and 8,000,000.

Mixtures of serum and antigen containing any considerable excess of either reactant yielded no precipitate. These zonal effects were not observed when the interfacial technic was used. With this technic the stratum of precipitate usually lies above the plane of junction of the reagents, *i.e.*, in the overlying antigen. We have not observed this with rabbit-sera. It could be attributed to the solubility of the precipitate in excess of equine antibody.

On each of the four large bleedings we made a fairly complete study of the relation between composition of precipitate and the proportion in which the reagents were mixed. Some precipitate was obtained even with several times the optimal amount of antigen. The maximal precipitate from a given volume of serum was usually obtained with an amount of antigen somewhat greater than the optimal (Dean and Webb)

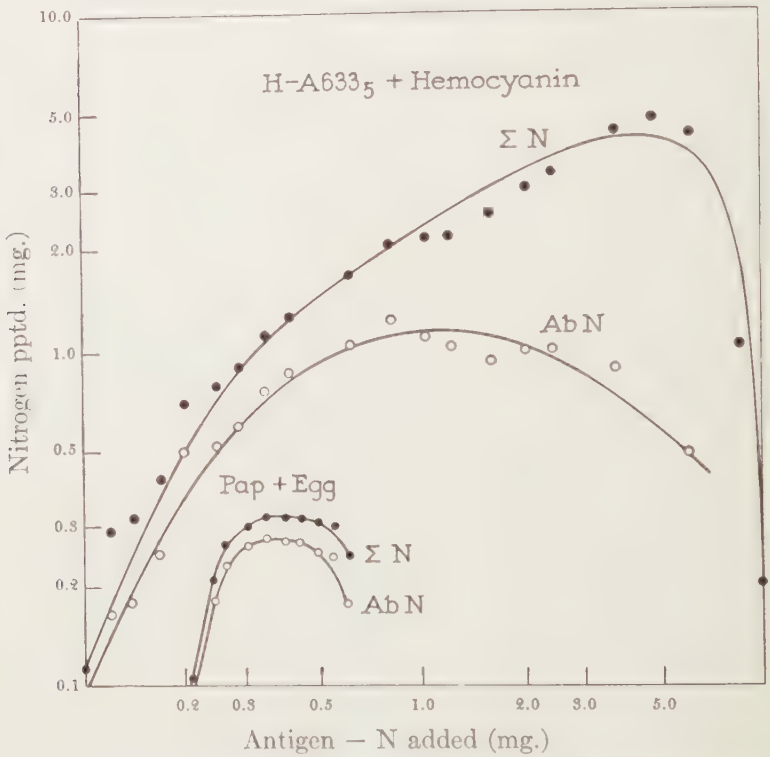

FIGURE 1. Total nitrogen and antibody-nitrogen precipitated from 1 ml. of fifth bleeding of horse 633 (antihemocyanin) and Papenheimer's horse 728 (anti-ovalbumin). (Done while serum was fresher than in FIGURES 2 and 3). Note concavity near top, ignored here, but evident also in FIGURES 2 and 3.

amount, although in the supernate little if any antigen remained (TABLE 5 and FIGURES 1, 2 and 3).

With the possible exception of the equivalence-zone ratios, the proportions of antibody and antigen in precipitates cannot be determined accurately by ordinary serological methods. Soluble compounds containing both antigen and antibody remain in the supernate, so it is impossible to assume that all of one reagent is precipitated and thus to

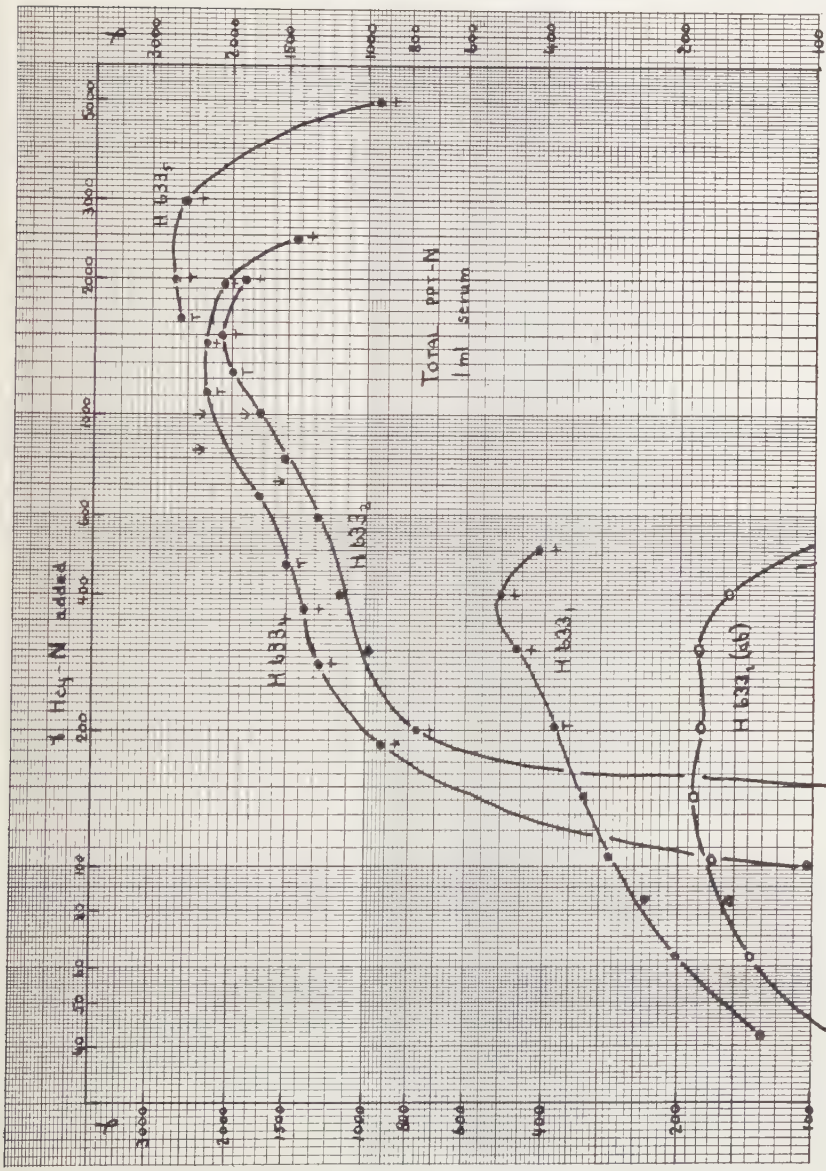

FIGURE 2. Total nitrogen precipitated from 1 ml. of different bleedings of horse 633 by varying amounts of antigen. + or T indicates excess antibody (or antigen) ↓ indicates constant-antibody (right) and constant-antigen optima.

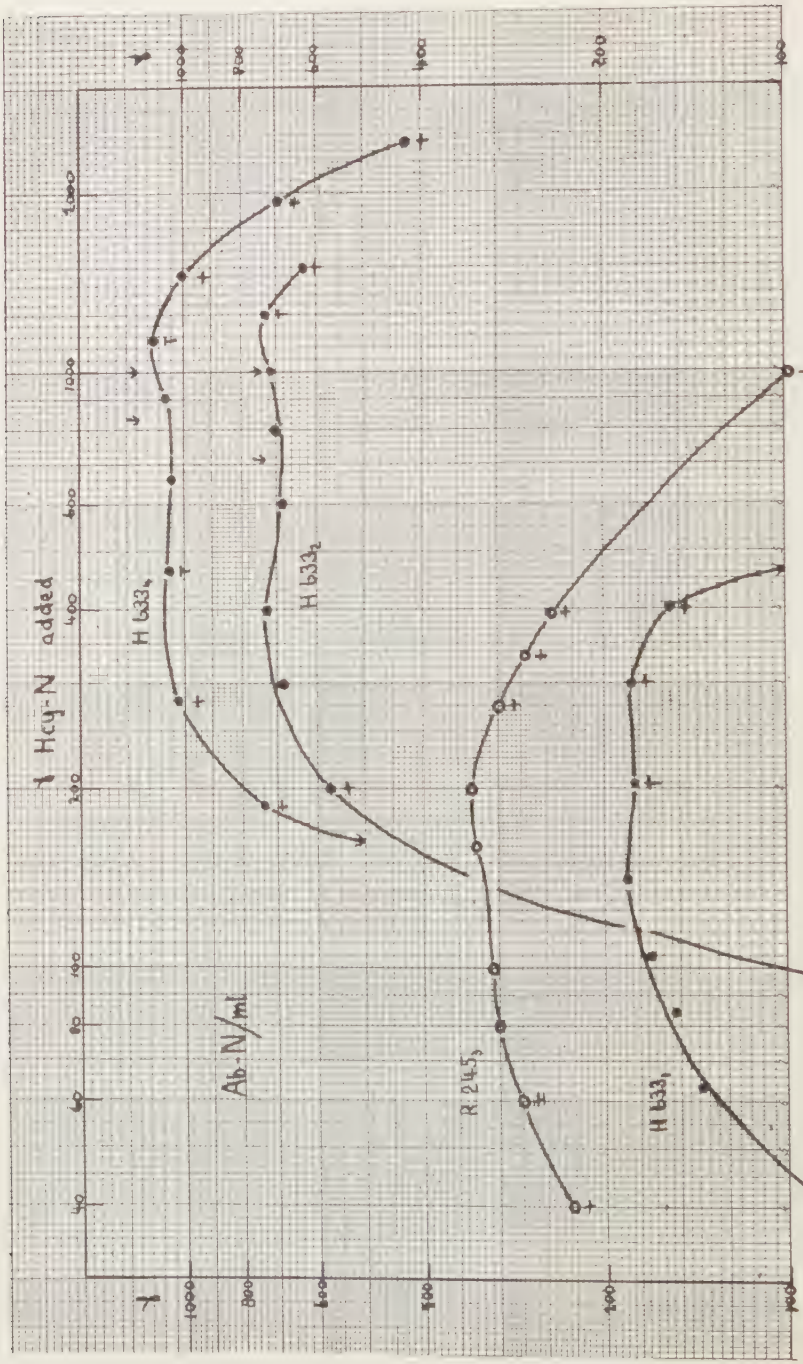

FIGURE 8. Antibody-nitrogen precipitated from 1 ml. of different bleedings of horse 633 and the third bleeding of rabbit 245, by varying amounts of antigen.

calculate the composition of the precipitate from measurements of nitrogen alone. The "linear-zone" method[9] proposed for determination of residual antigen was not satisfactory for these supernates even when there was a large excess of antigen. Therefore the ratio of antibody to antigen in the precipitates could only be determined by making analyses for copper as well as nitrogen. Preliminary determinations by this method showed that the ratio of antibody to antigen in the precipitate depended on the proportion in which the reagents were mixed, as usual. At the midpoint of the equivalence-zone it averaged about 1:2. From this point the ratio increased or decreased as the proportion of antibody to antigen in the mixtures was made greater or less. At the time of writing, sufficient analyses for copper are not at hand to permit more detailed statement.

The antiserum reacted strongly with hemocyanin from the closely related *B. carica*, and the quantitative data do not suggest any striking difference between the two antigens, although a detailed study of the similarity or possible identity of these two antigens has not yet been undertaken.

Rates of Precipitation

Different lots of hemocyanin varied considerably in the speed of flocculation with immune serum although the amounts of precipitate obtained were not very different. As in other systems, the rate of flocculation depended on the absolute concentration of reagents and on the ratio in which they were mixed. For any concentration of antibody, a certain concentration of antigen gave the most rapid flocculation (Dean-and-Webb optimum), the proportion between these concentrations being the same irrespective of the actual amounts used (TABLE 6). With concentrated reagents, virtually equal speeds of particulation were observed over a moderate range of proportions; with dilute reagents a "dead heat" was less often observed. If the optimum was determined by holding the concentration of antigen constant and varying the antibody (Ramon titration) the optimal-proportions point was not the same. As a consequence of this usual difference between the two optima, it was observed that flocculation in a mixture which was optimal according to the Dean-and-Webb titration could be speeded up by the addition of more antiserum.

Heat of Reaction

An attempt to measure directly the heat of an antibody-antigen reaction was made by Bayne-Jones.[10] It is generally considered[11] that

TABLE 6

"ROUGH" DEAN AND WEBB (ROWS) AND RAMON (COLUMNS) TITRATIONS.

$A633_5$ AND HEMOCYANIN DILUTIONS 1:1.5 TO THE POWER

ANTIGEN (ϕ = 7.5 mg. N/ml.)

Serum	ϕ	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	Times of flocculation in minutes																	
ϕ	< .5	< .5	< .5	< .5	< .5	< .5	1	5	34	>90								
1	>90	< .5	< .5	< .5	< .5	< .5	1.5	2	8	73	>90							
2			>90	3	< .5	< .5	1	1.5	4.5	17	>90							
3				>90	< .5	1.5	1.5	1.5	2	6.5	26.5	>90						
4					>90	28	5	5	2.5	5	12	34.5	>90					
5						>90	34	6.5	4	5	6.5	17.5	63	>90				
6							>90	64.5	12	7.5	7	11	24	57.5	>90			
7								>90	47	11	11	12.5	19	40.5	>90			
8									>90	35	17.5	12	16.5	19	48.5	>90		
9											>90	36.5	31	15.5	22.5	63	>90	
10												>90	59	24.5	30	45.5	71	>90

his result is much too high. With Kistiakowsky, *et al.*, we made measurements of the heat evolved when the hemocyanin reacted with serum (TABLE 7). In the region of antibody-excess *where no precipitate is formed*, a value of 3.0 calories per gram of antigen-nitrogen was found. As the molecular weight[12] of the antigen† is 6,800,000, this corresponds to about 3,300,000 calories per mol of antigen. It is believed that this

TABLE 7

HEAT EVOLVED ON MIXING HEMOCYANIN WITH NORMAL AND IMMUNE SERA

	Heat evolved	$-\Delta H$ kcal. x 10^{-3} per gm. hcy-N	$-\Delta H$ kcal. per mol hcy	Calc. $-\Delta H$ kcal. per mol antibody
Series 1.	*Cal.*			
(1) weak hcy + immune serum	+0.350			
(2) weak hcy + normal serum I	0.000			
(1) — (2)	+0.350	2.78	3027	35.6
Series 2.				
(1) weak hcy + immune serum	+0.405			
(2) weak hcy + normal serum II	+0.045			
(3) weak hcy + normal serum III	−0.090			
(1) −[(2) + (3)]/2	+0.428	3.58	3900	45.9
(4) strong hcy + immune serum	+0.144			
(5) strong hcy + normal serum IV	+0.045			
(4) − (5)	+0.099	0.11	116	2.7*

* Calculated for antibody reacting with the second addition of hemocyanin.

value is probably accurate to about 20 per cent. By extrapolation from analyses of specific precipitates, it was calculated that the above result corresponds to about 40,000 calories per mol of antibody. The magnitude of the result would presumably be different when the antibody and antigen were mixed in different proportions, and would probably be different for antigens of different molecular weights on account of the different numbers of specific combining groups.

† Since we began working with hemocyanins the estimates of some of their molecular weights have steadily risen and it is not certain that the upper limit has been reached. It has not always been clearly stated why these revisions have been made. The reader of some of our earlier work may multiply or divide our results by the factor that seems appropriate to him.

TABLE 8

COMPLEMENT-FIXATION

Serum $A633_5$	Antigen: Hemocyanin of *Busycon canaliculatum* γ hcy-N																					
ml.	γ ab-N	2500	1000	500	250	125	63	31	16	8	4	2	1	0.5	.25	.13	.06	.03	.015	.008	.004	Serum control
.04	38	–	4	4	**4***	**4**	**4**	4	3+	2	2	1	1	T	0	0	0	0	0	0	0	.16 **0**
.02	19	1	4–	4	4	4	**4**	**3**	2+	2	1	T	T	T	0	0	0	0	0	0	0	.08 0
.01	9	1	4–	4	4	4–	3+	2–	1–	T	0	0	0	0	0	0	0	0	0	0	0	.04 0
.005	4.5	T	4–	4	3+	3	1+	T	0	0	0	0	0	0	–	–	–	–	–	0	0	
.0033	3.1	–	4–	3	1	1–	T	0	0	0	0	0	0	0	–	–	–	–	–	0	0	
.0025	2.3	T	2	1+	T	T	T	0	0	0	0	0	0	0	–	–	–	–	–	0	0	
$H\ 633_1$																						
.04		–	4	4	4	4–	3–	1	T+	T+	T–	1–	2	3	3+	3	2	2	T+	0	0	.08 T
$R\ 245_3$																						.04 0
.01	3.3	–	4	4	4	4	4–	3+	3–	3	3+	4	4	4	4	4	4–	3	2	1	0	.04 1–
.007	2.4	–	4	4	4	4	4–	3+	2	2	2+	4–	4	4	4	4	4–	2+	1+	T	0	.02 T
.005	1.6	–	4	4	4	4–	3+	2	1	1	1	3	4–	4	4	4	3+	2	1	T	0	.01 0
.003	1	–	4	4	4–	2	2	1	T	T	T	1	2+	3+	4–	3+	3	1+	T	0	0	
.002	0.7	–	4–	4–	2	T	T	0	0	0	0	T	T	1	2	2	2	1	T	0	0	
.0015	0.5	–	3	2	1	T	0	0	0	0	0	0	0	1–	1	1	1	T	0	0	0	
Antigen control		T+	T	0	0																	

Alexin, 2 units. Sheep-cells, 0.1 ml. 10 per cent suspension sensitized with 2 units of lysin. Incubationary period, for fixation = 60′ 37 ; for hemolysis = 30′ 37°. 4 = complete fixation, T = trace, etc. Readings after refrigeration overnight.

* The figures in bold face indicate the mixtures that would give visible precipitation.

Complement-fixation

This system fixed guinea-pig complement strongly. Complete fixation occurred with quantities of antigen and of antibody that entirely inhibited precipitation. It also occurred with very small quantities of antigen; distinct fixation could be obtained with quantities as small as 1 γ of antigen-nitrogen; 30 γ was the smallest amount giving complete fixation (TABLE 8). This is in marked contrast to the behavior of a weaker rabbit-antihemocyanin ($R245_3$) that gave complete fixation with 0.13 γ and definite fixation with 0.008 γ antigen-N (1:125,000,000). The rabbit-antiserum exhibited two very distinct zones of fixation, having maxima at amounts of antigen-nitrogen of about 0.25 γ and over 1000 γ respectively. Serum from the first bleeding of the horse gave similar zones, but the zone of fixation with the small quantities of antigen was not observed in tests on the later bleedings. These zones are considered to indicate the presence of at least two qualitatively different antigenic components or determinants in our preparation of hemocyanin. Lyophilized serum was distinctly more anticomplementary than untreated serum.

Molecular ratios (*ab/ag*) in mixtures giving complete fixation (TABLE 8) are presented in TABLE 9. These ratios must be considered in attempts to formulate a mechanism of the complement-fixation reactions.

TABLE 9

MOLECULAR RATIO OF ANTIBODY TO ANTIGEN IN MIXTURES GIVING COMPLETE COMPLEMENT-FIXATION (FROM TABLE 8)

Serum	γ Antigen-nitrogen						
ml.	1000	500	250	125	63	31	16
0.04	1.8	3.6	7.2	14.4	29	57	
0.02	0.9	1.8	3.6	7.2	14		
0.01	0.45	0.9	1.8	3.6			
0.005	0.23	0.5					
0.0033	0.15						

The range of proportions observed to give complete fixation is seen to be 57/0.15 or a 380-fold range. Partial fixation occurred at ratios extending from 1824 to .05, or a 36,480-fold range.
For these estimates the m. w. of antibody was taken as 160,000 and of antigen as 8,000,000.

The heated antiserum already mentioned, which did not precipitate but which did inhibit precipitation, when tested for complement-fixation gave weaker positive reactions and did not inhibit or increase fixation by unheated serum (TABLE 10).

TABLE 10

FIXATION WITH HEATED ANTISERUM

Serum H 633_5	γ Antigen-nitrogen							
70° 30′	1000	500	250	125	63	31	—	0.5
.04 ml.	3+	2	1−	T+	T	0		
.02	2−	1+	T	T	T	0		
.01	T+	T	T	0	0	0		
.005	T	0	0	0	0	0		
Unheated .02	4	4	4	4	4	2+	2+	1−
+.02 70°	4	4	4	4	3+	3	2−	1−

Several workers have failed to obtain complement-fixation with horse-antisera.[13] Dean[14] observed fixation by mixtures of crude toxin and antitoxin, but realized that it might "be due to the interaction of other antigens" (than toxin). Fixation with meningococci[15] and pneumococcal protein[16] have been reported. We found that Pappenheimer's equine anti-ovalbumin gave definite fixation. These differences in fixative behavior of equine antisera do not seem readily explainable.

Passive Anaphylaxis

It is also known that most investigators excepting Bailey, *et. al.*[17] have been unable to sensitize the guinea pig passively with equine antiserum. We also failed to demonstrate passive anaphylaxis in guinea pigs previously injected with any dose of our serum within a wide range (1.0 to 0.005 ml.). For once the parallelism usually found between the fixative power of a serum and its ability to sensitize passively seems to be lacking. Typical fatal shock was induced in guinea pigs passively sensitized with rabbit-antihemocyanin containing an amount of antibody within the range of quantities given in the experiments with the equine serum.

SUMMARY

A powerful antiprotein was produced by injecting a horse with hemocyanin. Its serological behavior resembles strongly that of diphtheric antitoxin and equine anti-ovalbumin. This supports Pappenheimer's suggestion that equine antiproteins may have definite properties in common, distinguishing them from rabbit-antibodies and from equine anticarbohydrate. The heat of reaction was measured. The antiserum, especially when diluted, precipitated hemocyanin over a

relatively narrow range of quantity; it fixed guinea-pig complement strongly, but did not sensitize guinea pigs passively. The serum after moderate heating developed properties resembling those of "imperfect" or "univalent" antibody previously postulated by Heidelberger and by Pappenheimer.

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